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14. ABSTRACT The purpose was to investigate whether activation of 5-HT1B receptors within the ventral tegmental area (VTA) facilitated dopamine (DA) transmission in the ipsilateral nucleus accumbens (NACC) and potentiated ethanol-induced increases in NACC DA by 5-HT1B receptor-mediated GABA mechanisms. The data derived from microdialysis studies performed in rats and 5-HT1B receptor knockout mice, and those obtained from superfusion experiments consistently showed that activation of VTA 5-HT1B receptors facilitated mesolimbic DA transmission and enhanced the stimulatory effects of ethanol on mesolimbic DA neurons. These enhancements may be a consequence of additive effects resulting from indirect disinhibition of VTA DA neurons due to the receptor activation-induced inhibition of VTA GABA release. Moreover, the results showed that blockade of VTA 5-HT1B receptors attenuated ethanol-induced increases in mesolimbic DA transmission, suggesting the involvement of the receptor activation in mediating ethanol's DA activating effects. However, VTA GABA may not be involved in this 5-HT1B receptor-mediated effect since the data from in vivo and in vitro experiments showed that ethanol did not affect VTA GABA transmission. The data also showed that ethanol, when directly applied to VTA slices, increased somato-dendritic DA release, suggesting that the stimulatory effects of ethanol on VTA DA neurons observed in vivo may be associated, at least in part, with its direct actions within the VTA. Our data may explain in part why 5-HT1B receptors have been reported to be associated with alcohol abuse in both human genetics and in animal models, and to play a role in regulating alcohol voluntary intakes.

15. SUBJECT TERMS
Ethanol, Dopamine, Serotonin, 5-HT1B receptor, Ventral tegmental area, Nucleus accumbens, Rat, Knock-out mice, Microdialysis, Superfusion

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INTRODUCTION

The purpose of this project entitled "Ethanol and mesolimbic serotonin (5-HT)/dopamine (DA) interactions via 5-HT-1B receptors" was to investigate whether activation of 5-HT-1B receptors within the ventral tegmental area (VTA) facilitated DA transmission in the ipsilateral nucleus accumbens (NACC) and potentiated ethanol-induced increases in NACC DA by 5-HT-1B receptor-mediated GABA mechanisms. The scope of this project covered the following specific aims: (1) To determine the involvement of 5-HT-1B heteroreceptors on GABA terminals in the VTA in the modulation of GABA release in the VTA and DA release in the ipsilateral NACC, and its involvement in the neurochemical effect of acute ethanol in freely moving animals; (2) To compare the impact of 5-HT-1B receptor activation on DA transmission in the NACC and on ethanol's neurochemical effects between 5-HT-1B receptor knock-out (KO) mice and their counterparts wild-type (WT) mice; and (3) To determine the involvement of 5-HT-1B heteroreceptors on GABA terminals in the VTA in the modulation of DA and GABA releases in the VTA, and its involvement in the effect of ethanol in superfused VTA slices.

BODY

Specific Aim 1: To determine the involvement of 5-HT-1B heteroreceptors on GABA terminals in the VTA in the modulation of GABA release in the VTA and DA release in the ipsilateral NACC, and its involvement in the neurochemical effect of acute ethanol in freely moving animals

There were two hypotheses under Specific Aim 1: (1) Activation of 5-HT-1B receptors in the VTA decreased GABA release in this area and increased DA transmission in the ipsilateral NACC and (2) Activation and blockade of VTA 5-HT-1B receptors potentiated and attenuated ethanol's effects on DA transmission in the ipsilateral NACC, respectively.

The dual-probe microdialysis technique was used in awake and freely moving adult Sprague-Dawley rats. One probe was inserted into the VTA and the other in the ipsilateral NACC. Both probes were perfused with artificial cerebrospinal fluid (ACSF). For detailed description of the microdialysis and analytical procedures, please see Appendix 1.

1. Effects of infusion of CP 93129 into the VTA on extracellular DA concentrations in this region and in the ipsilateral NACC. In this experiment, ACSF containing three different concentrations of CP 93129 (20, 40 and 80 μM), a potent and selective 5-HT_{1B} receptor agonist[7,19], was administered via a probe into the VTA of three groups of rats for 60 min, respectively, and extracellular levels of DA in both the VTA and the ipsilateral NACC were monitored simultaneously. In another group of rats (the control group), ACSF was infused into the VTA for the same period as the drug groups and switching between syringes containing ACSF in this group was found to have no significant effects on the dialysate DA levels in the VTA (Fig 1) or in the ipsilateral NACC (Fig 2). As shown in Figs 1 and 2, administration of CP 93129 produced concentration-dependent increases in extracellular DA levels in both the VTA and NACC. In both regions, infusion of 80 μM of CP 93129 caused more pronounced increases than 20 μM (P = 0.015 and P = 0.045 at 40 and 60 min in Fig 1 and P = 0.046 at 20 min after drug infusion in Fig 2). The maximum increases of DA levels produced by 20, 40, and 80 μM of CP 93129 were 137%, 182%, and 242% of baseline in the VTA (Fig 1), and 160%, 181%, 217% of baseline in the NACC (Fig 2), respectively. The effects of 20 μM of CP 93129 on VTA DA did not reach statistical significance when compared with the control group.

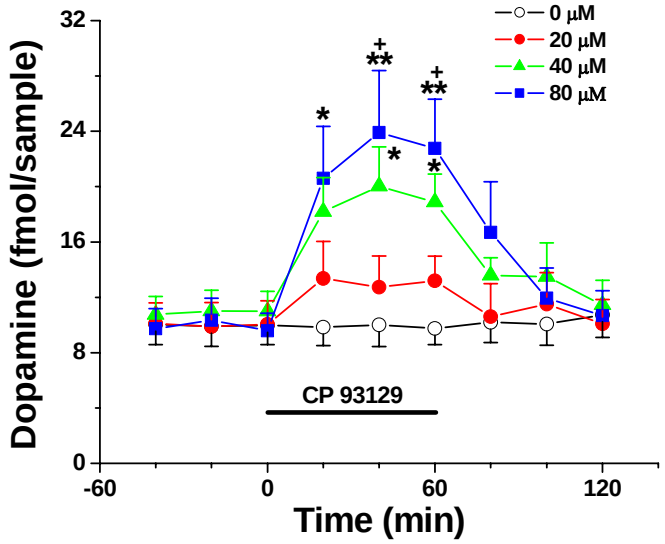

Fig 1. Effects of local infusion of CP 93129 into the VTA on extracellular DA in this region. CP 93129 (20, 40, and 80 μM) was administered via the probe into the VTA during the period indicated by the bar. Results are mean ± S.E.M from 6-7 animals. * P < 0.05, ** P < 0.01 as compared with the control (0 μM) group; $^{+}$ P < 0.05 as compared with the 20 μM group (two-way ANOVA followed by Tukey's tests).

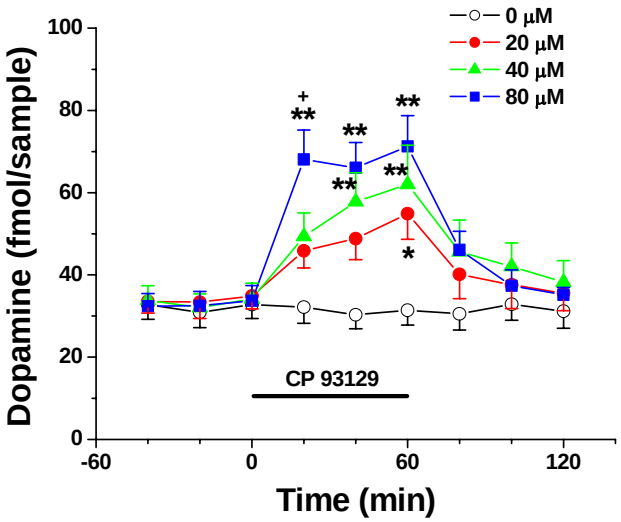

Fig 2. Effects of local infusion of CP 93129 into the VTA on extracellular DA in the ipsilateral NACC. CP 93129 (20, 40, and 80 μM) was administered via the probe into the VTA during the period indicated by the bar. Extracellular DA in the ipsilateral NACC was monitored by a second probe in this region. Results are mean ± S.E.M from 6-7 animals. * $P < 0.05$, ** $P < 0.01$ as compared with the control (0 μM) group; $^{+}$ $P < 0.05$ as compared with the 20 μM group (two-way ANOVA followed by Tukey's tests).

2. Effects of infusion of CP 93129 into the VTA on extracellular GABA concentrations in this region. As shown in Fig 3, infusion of ACSF alone (the control group) did not cause significant changes in GABA levels from VTA dialysates. Administration of CP 93129 at the concentration of 20 or 40 μM did not produce significant alterations in the level of VTA GABA either when compared with the control group although there were tendencies towards reductions following drug infusion. However, infusion of CP 93129 at the concentration of 80 μM caused extracellular GABA in the VTA to decrease by 37% of baseline ($P = 0.043$ and $P = 0.04$ at 20 and 60 min after drug application, respectively). Upon comparing the time course of VTA GABA with that of VTA DA or NACC DA after administration of 80 μM CP 93129, we found that the decrease of VTA GABA was correlated temporarily with the increase of DA in both the VTA and the NACC.

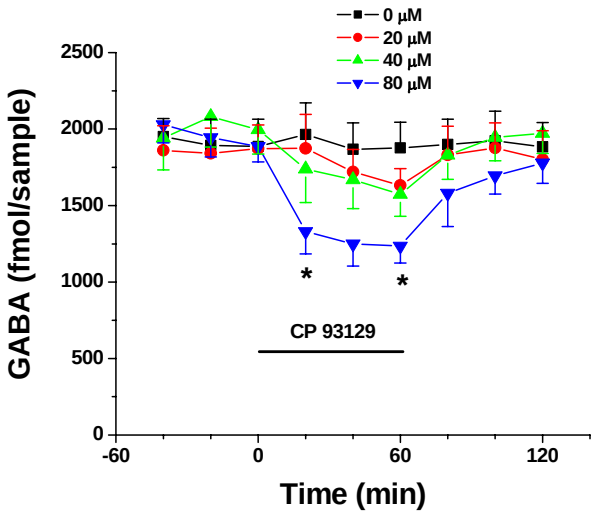

Fig 3. Effects of local infusion of CP 93129 into the VTA on extracellular GABA in this region. CP 93129 (20, 40, and 80 μM) was administered via the probe into the VTA during the period indicated by the bar. Results are mean ± S.E.M from 6-7 animals. * $P < 0.05$ as compared with the control (0 μM) group (two-way ANOVA followed by Tukey's tests).

3. Effects of WAY-100635, SB 216641, or BRL 15572 on intra-tegmental CP 93129 (80 μM)-induced DA release in the VTA and the ipsilateral NACC. In these experiments, WAY 100635 (a 5-HT_{1A} receptor antagonist), SB 216641 (a 5-HT_{1B} receptor antagonist), and BRL 15572 (a 5-$HT_{1D/1A}$ receptor antagonist) were used. All these drugs were infused into the VTA at the concentration of 10 μM for 40 min alone and then co-infused with CP 93129 (80 μM) for another 60 min, respectively.

In separate groups of rats infusion of WAY 100635, SB 216641, or BRL 15572 at the concentration of 10 μM into the VTA for 2 h did not significantly alter extracellular DA levels in either the VTA or the ipsilateral NACC (data not shown).

Figs 4 and 5 show comparisons of intra-tegmental CP 93129 (80 μM)-induced accumbal DA release in the presence and absence of WAY-100635, SB 216641, or BRL 15572. It can be seen from these figures, the CP 93129-induced DA releases in the VTA and the NACC were all significantly attenuated by co-infusion of SB 216641. In the presence of SB 216641, CP 93129 (80 μM)-induced DA outputs in the VTA (Fig 4) and NACC (Fig 5) were all significantly lower than those in the absence of SB 216641 ($P < 0.05$, two-way ANOVA followed by Tukey's tests). However, co-administration of WAY-100635 or BRL 15572 had no significant effects on the CP 93129-induced DA release in either the VTA or the NACC.

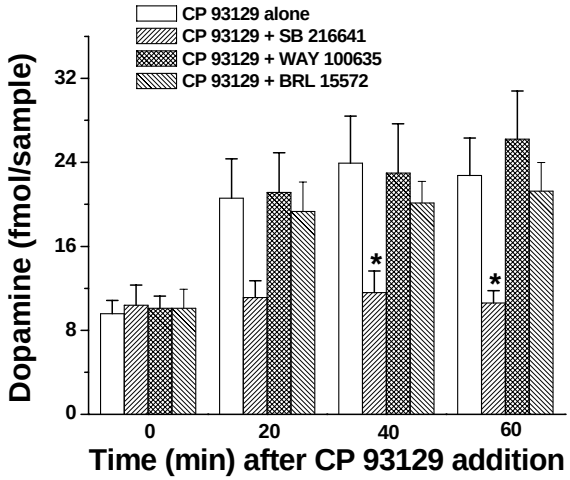

Fig 4. Comparisons of CP 93129-induced increases of extracellular VTA DA in the presence and absence of WAY-100635, SB 216641, or BRL 15572. WAY-100635 (10 μM), SB 216641 (10 μM), and BRL 15572 (10 μM) were administered through the probe into the VTA for 40 min, and then co-infused with CP 93129 (80 μM) for another 60 min, respectively. Results are mean ± S.E.M from 6-7 animals. The data of the group of CP 93129 (80 μM) alone were obtained from Fig 1. ***** $P <$ 0.05 as compared with the group of CP 93129 alone (two-way ANOVA followed by Tukey's tests).

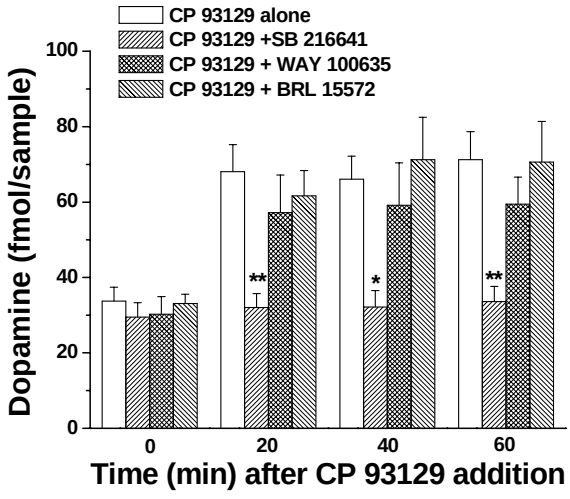

Fig 5. Comparisons of CP 93129-induced increases of extracellular NACC DA in the presence and absence of WAY-100635, SB 216641, or BRL 15572. WAY-100635 (10 μM), SB 216641 (10 μM), and BRL 15572 (10 μM) were administered through the probe into the VTA for 40 min, and then co-infused with CP 93129 (80 μM) for another 60 min, respectively. Extracellular DA in the ipsilateral NACC was monitored by a second probe in this region. Results are mean ± S.E.M from 6-7 animals. The data of the group of CP 93129 (80 μM) alone were obtained from Fig 2. ***** $P < 0.05$, ****** $P < 0.01$ as compared with the group of CP 93129 alone (two-way ANOVA followed by Tukey's tests).

4. Effects of WAY-100635, SB 216641, or BRL 15572 on intra-tegmental CP 93129 (80 μM)-induced reductions of GABA release in the VTA. In separate groups of rats infusion of WAY 100635, SB 216641, or BRL 15572 at the same concentration used into the VTA for 2 h did not significantly alter extracellular GABA levels in the VTA (data not shown). As shown in Fig 6, administration of SB 216641, but not WAY 100635 or BRL 15572, antagonized the effects of intra-tegmental CP 93129 on VTA GABA.

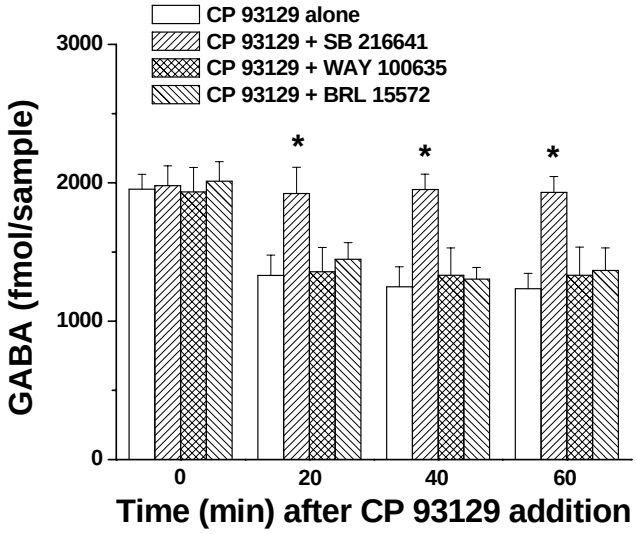

Fig 6. Comparisons of CP 93129-induced decreases of extracellular VTA GABA in the presence and absence of WAY-100635, SB 216641, or BRL 15572. WAY-100635 (10 μM), SB 216641 (10 μM), and BRL 15572 (10 μM) were administered through the probe into the VTA for 40 min, and then co-infused with CP 93129 (80 μM) for another 60 min, respectively. Results are mean ± S.E.M from 6-7 animals. The data of the group of CP 93129 (80 μM) alone were obtained from Fig 3. ***** $P < 0.05$ as compared with the group of CP 93129 alone (two-way ANOVA followed by Tukey's tests).

The data presented above show that local application of CP 93129 into the VTA increased not only somato-dendritic DA release in this region but also DA release from nerve terminals in the NACC, consistent with the excitation of mesolimbic DA neurons.

To assess the involvement of 5-HT_{1B} receptors in CP 93129's actions, WAY-100635 (a 5-HT_{1A} receptor antagonist), SB 216641 (a 5-HT_{1B} receptor antagonist), and BRL 15572 (a 5-$HT_{1D/1A}$ receptor antagonist) were used. If 5-HT_{1A} receptors are involved in the actions of CP 93129, WAY 100635 and BRL 15572 would to some degree antagonize the effects of CP 93129 on VTA DA or NACC DA. However, the data presented here show that administration of neither WAY 100635 nor BRL 15572 antagonizes the effects of intra-tegmental CP 93129 on extracellular DA in either the VTA or the NACC. These results are in opposition with the involvement of 5-HT_{1A} receptors in the observed CP 93129's effects. The present data also show that intra-tegmental CP 93129-induced augmentations of VTA DA and NACC DA were significantly antagonized only by local infusion of SB 216641 but not BRL 15572. These data are consistent with the involvement of 5-HT_{1B} receptors but not 5-HT_{1D} receptors in CP 93129's actions. Taken together, the present results suggest that increased activity of mesolimbic DA neuron following intra-tegmental administration of CP 93129 may be associated with the drug-induced activation of 5-HT_{1B} receptors within the VTA.

The present study also shows that local infusion of CP 93129 at the concentration of 80 μM caused a decrease of extracellular GABA in the VTA. This effect of CP 93129 was also antagonized by local administration of SB 216641 but not by either WAY 100635 or BRL 15572. The results suggest that it is activation of VTA 5-HT_{1B} receptors that may be associated with the drug-induced reduction of GABA release in this region. These data are in good agreement with our previous in vitro studies showing that activation of 5-HT_{1B} receptors by CP 93129 or RU 24969 inhibited high potassium-evoked [^{3}H]GABA release from rat VTA slices in a concentration-dependent fashion[33]. Chadha et al.[4] also reported that CP 93129 produced a concentration-dependent inhibition of high potassium-evoked [^{3}H]GABA release from rat globus pallidus slices. Since VTA DA neurons are under inhibitory

GABAergic controls, reductions of VTA GABA release by 80 μM of CP 93129 may account for the drug-induced increase of VTA DA neuronal activity.

The present results show that administration of CP 93129 at lower concentrations (i.e., 20 or 40 μM) did not significantly alter GABA levels but did increase DA concentrations in the VTA and the NACC. The results suggest that, in addition to indirect disinhibition of VTA DA neurons resulting from decreased GABAergic control, other mechanisms may also be involved in CP 93129-induced augmentations of VTA DA neuronal activity.

In summary, the results suggest that activation of VTA 5-HT_{1B} receptors is associated with CP 93129-induced activation of mesolimbic DA neurons. This conclusion is based on the data that intra-tegmental CP 93129 increased DA release in both the VTA and the NACC and that the increase of the DA release was antagonized by the 5-HT_{1B} receptor antagonist but not by the 5-HT_{1A} or 5-HT_{1D} receptor antagonist. The results also suggest that the 5-HT_{1B} receptor-mediated inhibition of VTA GABA release may contribute, at least in part, to the observed activation of mesolimbic DA neurons. This conclusion is drawn from the data that intra-tegmental CP 93129 concomitantly caused a reduction of VTA GABA, an effect that was also blocked by the 5-HT_{1B} receptor antagonist. In addition to this indirect disinhibition of VTA DA neurons resulting from decreased GABAergic control, other mechanisms may also be involved in CP 93129-induced augmentation of mesolimbic DA transmission, particularly under lower concentrations of the drug. This speculation is based on the data that CP 93129 increased DA release at all concentrations tested but significantly decreased VTA GABA release only at a higher concentration. All these data have been published in *Brain Research* (1021: 82-91, 2004)[34] (please see Appendix 1).

5. Effects of systemic ethanol on extracellular DA and GABA in the VTA, and DA in the ipsilateral NACC. After DA and GABA in the VTA, and DA in the ipsilateral NACC were stable, ethanol (1 and 2 g/kg) or saline was injected ip and microdialysis was continued for another 2 h. As shown in Fig 7, intraperitoneal (ip) injection of saline had no significant effects on dialysate levels of DA in either the VTA or the ipsilateral NACC. However, administration of ethanol at the doses of 1 and 2 g/kg significantly increased extracellular DA concentrations by ~38% and ~56% of baseline in the VTA ($P < 0.01$ as compared with the saline group, the upper panel of Fig 7), and ~51% and ~ 67% of baseline in the NACC ($P < 0.01$ as compared with the saline group, the lower panel of Fig 7), respectively. As can be seen from this figure, the maximum increases in extracellular DA concentrations in the VTA were temporally correlated with those in the NACC after administration of ethanol at both doses.

As can be seen from Fig 8, administration of ethanol at the doses of 1 or 2 g/kg did not cause any significant changes in dialysate GABA levels as compared with the saline group, suggesting that acute ethanol may not affect extracellular GABA in the VTA under the present experimental conditions. Since no measurable changes in VTA dialysate GABA were detected after administration of ethanol at the doses of 1 or 2 g/kg, GABA contents in the VTA dialysates were no longer assayed in the following experiments.

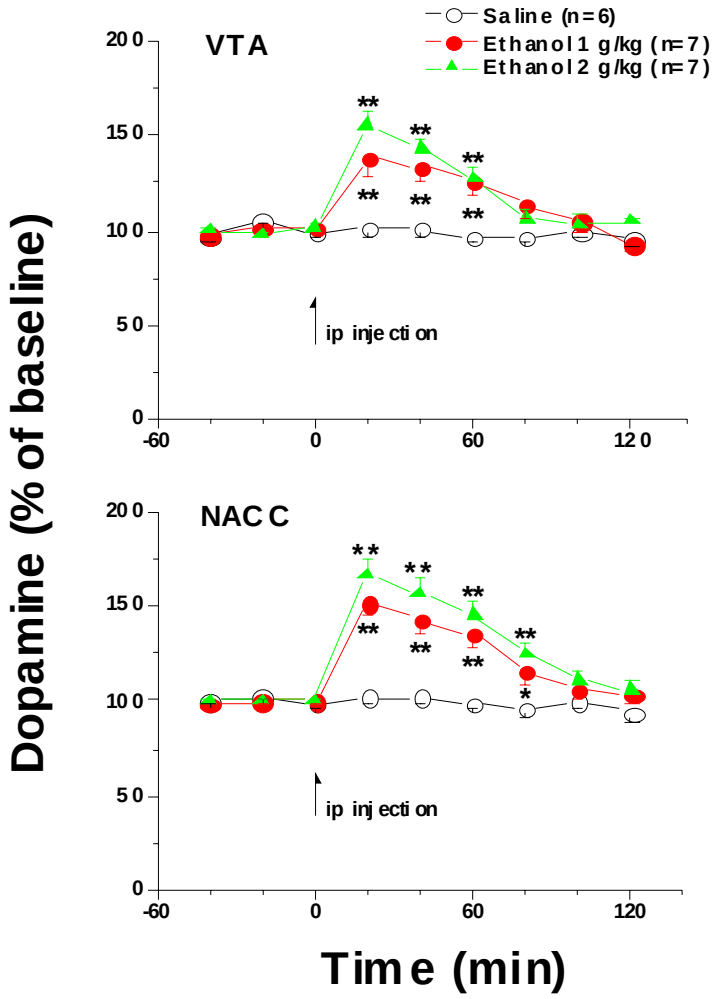

Fig 7. Effects of acute ethanol on extracellular DA in the VTA (the upper panel) and the ipsilateral NACC (the lower panel). Microdialysis probes placed in the VTA and the NACC were perfused with ACSF simultaneously. Saline or ethanol (1 and 2 g/kg) was administered by ip injection indicated by the arrow. Results are mean ± SEM from six to seven animals. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the saline group (two-way ANOVA followed by Tukey's tests). The basal values (fmol/sample) of extracellular DA in the VTA and NACC were: 9.04 ± 1.32 and 29.09 ± 3.09 (the saline group, n = 6), 10.99 ± 1.04 and 34.39 ± 2.72 (the 1 g/kg ethanol group, n = 7), and 8.94 ± 0.72 and 28.24 ± 2.30 (the 2 g/kg ethanol group, n = 7), respectively. There were no statistically significant differences in basal DA in the VTA or the NACC among the ethanol and saline groups.

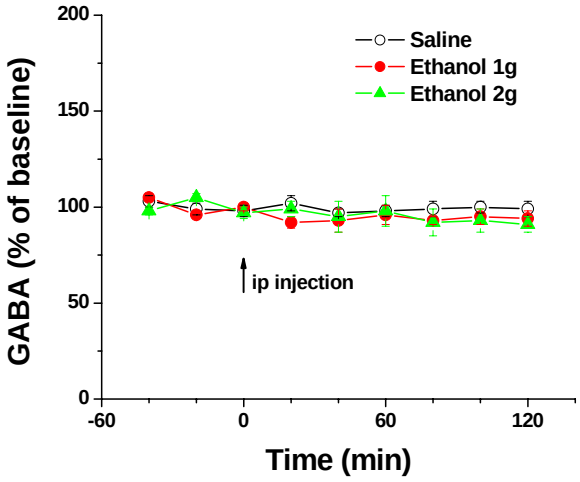

Fig 8. Effects of acute ethanol on extracellular GABA in the VTA. Microdialysis probes placed in both the VTA and the ipsilateral NACC were perfused with ACSF simultaneously. Saline or ethanol (1 and 2 g/kg) was administered by ip injection indicated by the arrow. Results are mean ± SEM from six to seven animals. There were no statistically significant differences among the ethanol and saline groups. Basal GABA levels (fmol/sample) in the VTA dialysate were: 1729.95 ± 141.63 (the saline group, n = 6), 1791.73 ± 107.41 (the 1 g/kg ethanol group, n = 7), and 1647.43 ± 97.81 (the 2 g/kg ethanol group, n = 7).

6. Effects of infusion of 5-HT-1 receptor antagonists into the VTA on ethanol-induced DA release in this region and in the ipsilateral NACC.

In these experiments, SB 216641, BRL 15572, or WAY 100635 was infused into the VTA 20 minutes before ethanol administration and remained throughout the experiments. All these antagonists were used at the concentration of 10 μM in ACSF.

Figs 9 and 10 show comparisons of ethanol (1 and 2 g/kg)-induced DA release in the VTA and the NACC in the presence and absence of SB 216641, WAY 100635, or BRL 15572. As shown in these figures, ethanol-induced DA releases, except those occurred in the VTA after 1 g/kg of ethanol (the upper left of Fig 9), were all significantly attenuated by co-administration of SB 216641. In the presence of SB 216641, ethanol (1 and 2 g/kg)-induced NACC DA release (the upper right of Figs 9 and 10) and ethanol (2 g/kg)-induced VTA DA release (the upper left of Fig 10) were all significantly lower than those in the absence of SB 216641. However, co-administration of WAY 100635 or BRL 15572 had no significant effects on ethanol-induced DA release in either the VTA or the NACC.

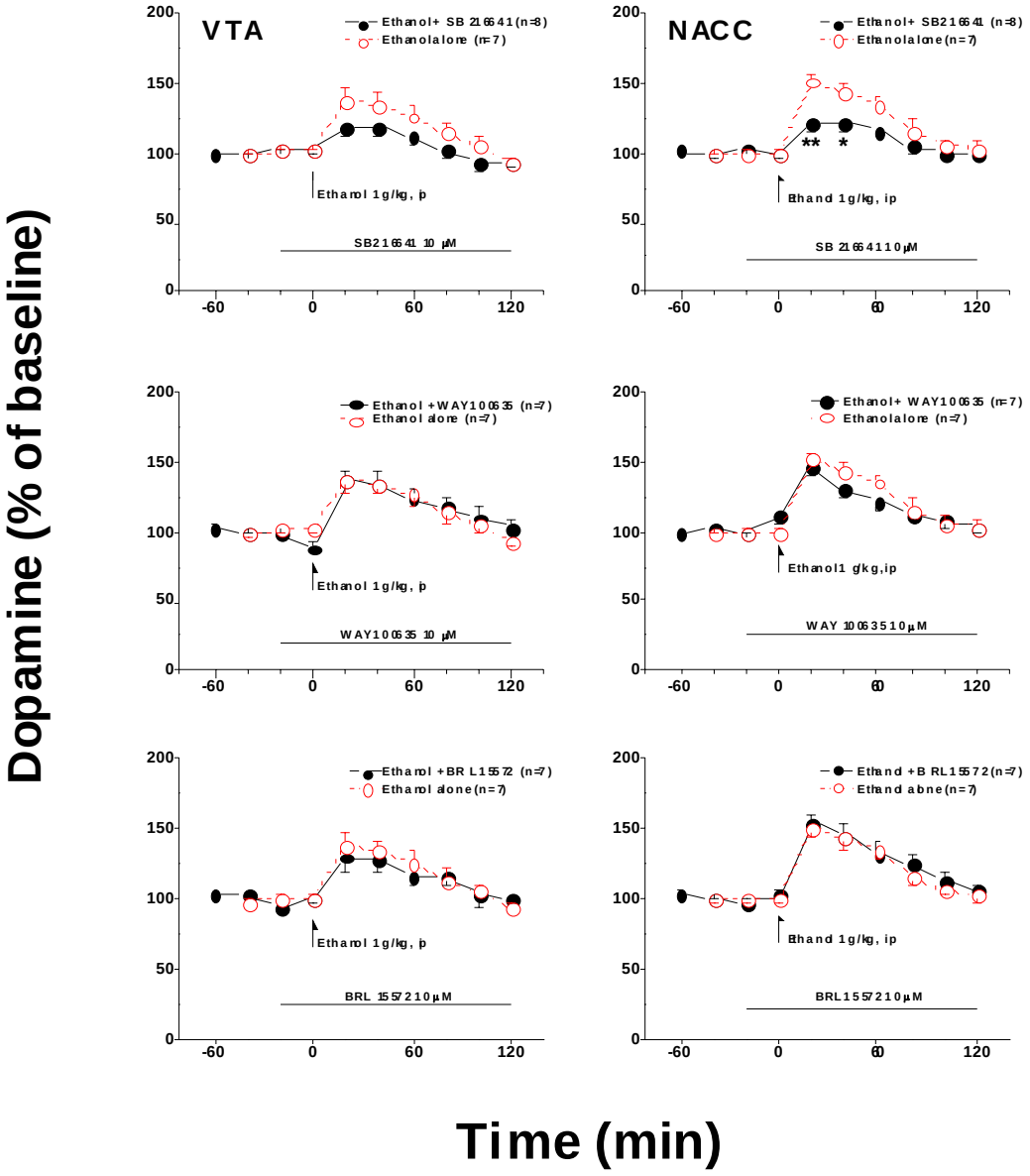

Fig 9. Comparisons of ethanol (1 g/kg)-induced DA release in the VTA (the left panel) and the ipsilateral NACC (the right panel) in the presence and absence of SB 216641 (upper), WAY 100635 (middle), or BRL 15572 (lower). SB 216641 (10 μM), WAY 100635 (10 μM), or BRL 15572 (10 μM) was infused into the VTA 20 min before ethanol (1 g/kg, ip) administration and remained throughout the experiments. Results are mean ± SEM from seven to eight animals. The data of the ethanol alone group were obtained from Fig 7. The basal DA levels (fmol/sample) in the VTA and the NACC were: 10.99 ± 1.04 and 34.39 ± 2.72 (the ethanol alone group, n=7), 11.16 ± 0.81 and 46.78 ± 4.84 (the ethanol + SB 216641 group, n=8), 10.31 ± 1.18 and 36.92 ± 3.96 (the ethanol + WAY 100635 group, n=7), and 12.32 ± 1.37 and 37.71 ± 3.47 (the ethanol + BRL 15572 group, n=7), respectively. * $P < 0.05$, ** $P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests).

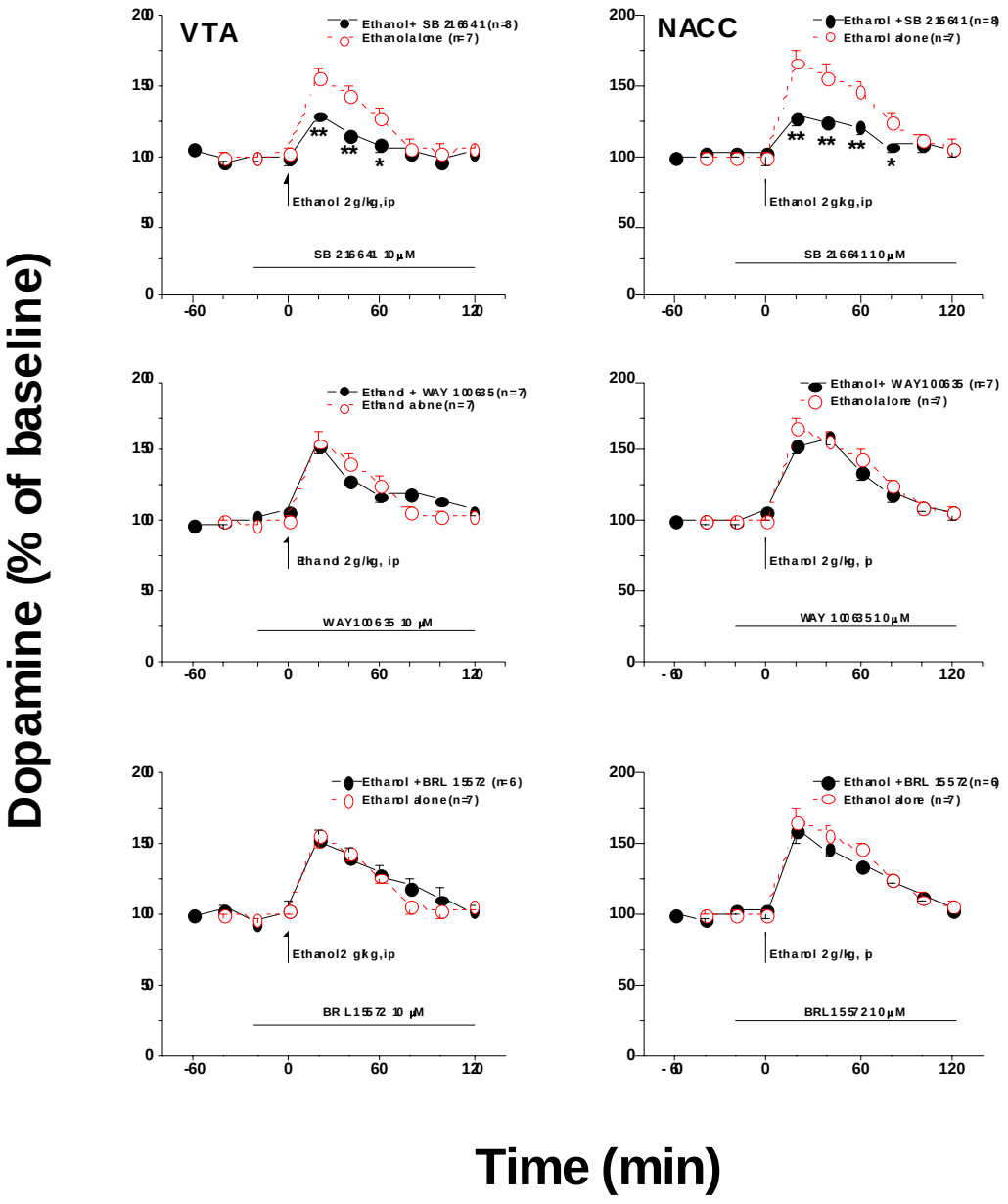

Fig 10. Comparisons of ethanol (2 g/kg)-induced DA release in the VTA (the left panel) and the ipsilateral NACC (the right panel) in the presence and absence of SB 216641 (upper), WAY 100635 (middle), or BRL 15572 (lower). SB 216641 (10 μM), WAY 100635 (10 μM), or BRL 15572 (10 μM) was infused into the VTA 20 min before ethanol (2 g/kg, ip) administration and remained throughout the experiments. Results are mean ± SEM from six to eight animals. The data of the ethanol alone group were obtained from Fig 7. The basal DA levels (fmol/sample) in the VTA and the NACC were: 8.94 ± 0.72 and 28.24 ± 2.30 (the ethanol alone group, n=7), 8.24 ± 0.83 and 26.83 ± 2.53 (the ethanol + SB 216641 group, n=8), 9.25 ± 1.04 and 28.79 ± 2.20 (the ethanol + WAY 100635 group, n=7), and 9.06 ± 0.93 and 27.64 ± 1.85 (the ethanol + BRL 15572 group, n=6), respectively. * $P < 0.05$, ** $P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests).

7. Effects of infusion of CP 94253 into the VTA on ethanol (1 and 2 g/kg)-induced DA release in the ipsilateral NACC.

These experiments were designed to investigate further the involvement of VTA 5-HT_{1B} receptors in modulation of the effects of ethanol on DA release in the NACC. In these experiments CP 94253 (10 μM), another 5-HT_{1B} receptor agonist, was infused into the VTA 20 minutes before ethanol injections and remained throughout the experiments. In a separate group of rats, perfusion of the VTA with CP 94253 (10 μM in ACSF) followed by the saline injection did not significantly alter extracellular DA levels in the ipsilateral NACC (Figs 11 and 12). As shown in Figs 11 and 12, however, administration of CP 94253 significantly changed the time course of extracellular DA concentrations following ethanol administration. In the absence of CP 94253, extracellular DA in the NACC increased rapidly to the maximum level after administration of ethanol at the doses of 1 (Fig 11) and 2 g/kg (Fig 12), then declined and reached the control level at 80 min after ethanol injection. However, in the presence of CP 94253, extracellular DA still remained significantly high levels as compared with either the saline or the ethanol alone group at 80 – 120 min after administration of ethanol at the both doses (Figs 11 and 12). The results indicated that the administration of CP 94253 significantly prolonged the effects of ethanol (1 and 2 g/kg) on the extracellular DA in the NACC although the maximum increases of NACC DA after co-administration of CP 94253 and ethanol did not significantly differ from those after administration of ethanol alone.

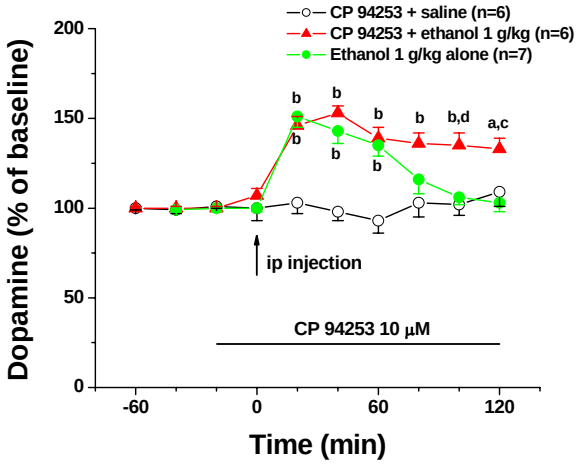

Fig 11. Effects of co-administration of CP 94253 on ethanol (1 g/kg)-induced NACC DA release. CP 94253 (10 μM) was infused into the VTA through a probe as indicated by the bar. Ethanol (1 g/kg) or saline was injected ip as indicated by the arrow. Extracellular DA in the ipsilateral NACC was measured by a second probe in this region. The data of the ethanol alone group was obtained from Fig 7. [a] $P < 0.05$, [b] $P < 0.01$ as compared with the CP 94253 + saline group; [c] $P < 0.05$, [d] $P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests). The basal DA levels (fmol/sample) were: 34.39 ± 2.72 (the ethanol alone group), 37.02 ± 3.64 (the CP 94253 + saline group), and 35.97 ± 3.73 (the CP 94253 + ethanol group).

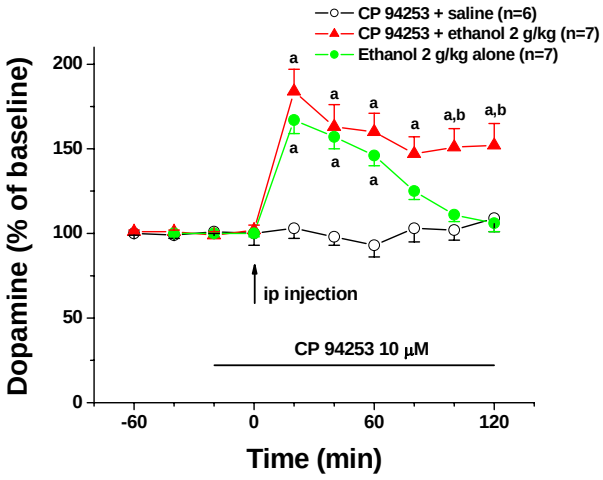

Fig 12. Effects of co-administration of CP 94253 on ethanol (2 g/kg)-induced NACC DA release. CP 94253 (10 μM) was infused into the VTA through a probe as indicated by the bar. Ethanol (2 g/kg) or saline was injected ip as indicated by the arrow. Extracellular DA in the ipsilateral NACC was measured by a second probe in this region. The data of the ethanol alone group were obtained from Fig 7. The data of the CP 94253 + saline group were obtained from Fig 11. [a] $P < 0.01$ as compared with the CP 94253 + saline group; [b] $P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests). The basal DA levels (fmol/sample) were: 28.24 ± 2.30 (the ethanol alone group), 37.02 ± 3.64 (the CP 94253 + saline group), and 34.99 ± 3.46 (the CP 94253 + ethanol group).

Consistent with the findings by Kohl et al.[14], the present data show that systemic administration of ethanol at the doses of 1 and 2 g/kg increases extracellular DA concentrations not only in the NACC but also in the VTA in the same animal. Simultaneous elevations in the extracellular levels of DA in both cell body and terminal areas suggest that systemic administration of ethanol increases the firing rate of VTA DA neurons. Interestingly, administration of nicotine, a drug that increases mesolimbic DA transmission by similar mechanisms as ethanol[20], also increased both somatodendritic DA release in the VTA and synaptic DA release in the NACC[36]

The data presented here show that administration of neither WAY 100635 nor BRL 15572 into the VTA antagonizes the effects of systemic ethanol on extracellular DA in either the VTA or the NACC. These results are in opposition with the involvement of activation of VTA 5-HT_{1A} receptors in ethanol's effects. The present data also show that systemic ethanol-induced augmentations of VTA DA and NACC DA were significantly attenuated by local administration of SB 216641 but not by BRL 15572. These data are consistent with the involvement of activation of VTA 5-HT_{1B} receptors, but not VTA 5-HT_{1D} receptors, in mediating the ethanol-induced excitation of VTA DA neurons.

In order to further investigate the involvement of VTA 5-HT_{1B} receptors in ethanol-induced activation of mesolimbic DA neurons, the 5-HT_{1B} receptor agonist CP 94253 was used. Compared with CP 93129, CP 94253 has higher affinities for 5-HT_{1B} receptors (IC_{50} values for 5-HT_{1B} receptors of CP 94253 and CP 93129 are 2 nM and 15 nM, respectively[7,13,19]). The data presented here showed that local infusion of 10 μM CP 94253 into the VTA did not significantly alter extracellular DA concentrations in the ipsilateral NACC. This result suggests that this concentration of CP 94253 may not cause sufficient activation of VTA 5-HT_{1B} receptors to produce significant increases in the activity of mesolimbic DA neurons under the present experimental conditions. However, this concentration of CP 94253 did enhance the effects of ethanol on extracellular DA in the NACC. The present data show that the treatment with CP 94253 significantly prolongs the effects of ethanol although it does not significantly enhance ethanol's peak effects. The lack of further increases of ethanol's peak effects by CP 94253 may be a result of the VTA 5-HT_{1B} receptors already being maximally (or near maximally) activated by ethanol-evoked 5-HT, consequently, leading to little potential for further activation by CP 94253. Together with the data obtained with the 5-HT_{1B} receptor antagonist, the results support the suggestion that activation of 5-HT_{1B} receptors within the VTA may contribute to systemic ethanol-induced increases of mesolimbic DA neuronal activities.

The previous microdialysis studies (Fig 3) show that activation of VTA 5-HT_{1B} receptors by CP 93129 inhibits GABA neurotransmission within the VTA that may result in disinhibition of mesolimbic DA neurons. These findings prompted us to speculate that the 5-HT_{1B} receptor-mediated inhibition of VTA GABA transmission may contribute to the stimulatory effect of ethanol on VTA DA neurons. Therefore, potential effects of systemic ethanol on VTA GABA were examined in this study. Unfortunately, the data presented in Fig 8 indicated that administration of ethanol at the dose of 1 or 2 g/kg did not significantly alter extracellular GABA in the VTA but did increased DA concentrations in both the VTA and the NACC (Fig 7). These results do not support our speculation.

However, it should be pointed out that microdialysis requires long sampling time, and as such can only readily quantify substantial changes in release of neurotransmitters that maintained for a period of time. It is possible that administration of ethanol evokes a transient change in VTA GABA levels that is masked in a 20-min sample. In addition, previous studies suggest that GABA levels monitored by microdialysis probes may derive from both the non-neuronal and the neuronal pools. Consequently, it is also possible that potential alterations in neuronal GABA release resulting from ethanol administration may only cause a small change in total extracellular GABA that cannot be detected by our HPLC system. As a result, an inability to detect measurable changes in VTA GABA release following ethanol does not necessarily mean that VTA GABA is not a target of acute ethanol.

In summary, the present results indicate that blockade of VTA 5-HT_{1B} receptors attenuates the ethanol-induced increases in extracellular DA concentrations in both the VTA and the

ipsilateral NACC. The data support the suggestion that activation of VTA 5-HT$_{1B}$ receptors may be involved in part in mediating the stimulatory effects of ethanol on mesolimbic DA neurons. Although the 5-HT$_{1B}$ receptor activation-induced inhibition of VTA GABA release may contribute to the receptor agonist-induced activation of mesolimbic DA neurons (see above), the data presented here do not support the involvement of VTA GABA mechanisms in the 5-HT$_{1B}$ receptor-mediated modulation of the ethanol's effects since no significant changes in VTA GABA levels are detected after ethanol administration. The present data also show that additional activation of VTA 5-HT$_{1B}$ receptors by the 5-HT$_{1B}$ receptor agonist may enhance the ethanol-induced increase in mesolimbic DA transmission. All these data have been published in *Brain Research*[35] (1060: 126-137, 2005; also see Appendix 2).

Conclusions about the specific aim 1: The results from the above experiments support the following hypotheses that were set under Specific Aim 1: (1) activation of 5-HT-1B receptors in the VTA decreases GABA release in this area and increases DA transmission in the ipsilateral NACC and (2) activation and blockade of VTA 5-HT-1B receptors potentiate and attenuate ethanol's effects on DA transmission in the ipsilateral NACC, respectively. Further studies are warranted to elucidate the mechanisms by which VTA 5-HT$_{1B}$ receptors are involved in mediating the stimulatory effects of ethanol on mesolimbic DA neurons.

Specific Aim 2: To compare the impact of 5-HT-1B receptor activation on DA transmission in the NACC and on ethanol's neurochemical effects between 5-HT-1B receptor knock-out (KO) mice and their counterparts wild-type (WT) mice.

There were three hypotheses under Specific Aim 2: (1) Facilitation of NACC DA transmission by activation of the 5-HT$_{1B}$ receptor is absent in 5-HT$_{1B}$ receptor KO mice; (2) Systemic ethanol-induced increases in NACC DA is more pronounced in WT mice than in KO mice, and (3) Potentiation of the effects of ethanol on NACC DA transmission by activation of the 5-HT$_{1B}$ receptor is absent in 5-HT$_{1B}$ receptor KO mice.

In the following experiments one-probe microdialysis was performed in mice with the probe being inserted in the NACC. For detailed description of the microdialysis and analytical methods, please see Appendix 3.

8. Effects of systemic administration of RU 24969 on extracellular DA concentrations in the NACC of the KO and WT mice.

After basal DA in the NACC was stable, saline or RU 24969 (0.5 and 1 mg/kg), a 5-HT$_{1B/1A}$ receptor agonist[7], was injected ip to the KO or WT mice. The dose (0.5 and 1 mg/kg) of RU 24969 was chosen as administration of RU 24969 at this dose range increased NACC DA in rats[2] and in C57BL/6J mice in our pilot study.

As shown in Fig 13, administration of RU 24969 at the doses of 0.5 and 1 mg/kg did not produce significant changes in extracellular NACC DA in the KO mice as compared with the saline group (Panel A). In contrast, administration of RU 24969 at the same doses produced a dose-related increase in extracellular NACC DA in the WT mice (Panel B). There was a slight increase in NACC DA in the WT mice after 0.5 mg/kg of RU 24969 but the effect did not reach statistical significances as compared with the saline group (Panel B). However, administration of RU 24969 at the higher dose (1 mg/kg) produced statistically significant increases in NACC DA in the WT mice (Panel B). As shown in Panel B, after administration of 1 mg/kg of RU 24969 extracellular NACC DA levels in the WT mice increased gradually, and statistically significant increases occurred from the time-points of 40 (as compared with the saline group) and 80 min (as compared with the 0.5 mg of RU 24969 group) until the end of the experiment.

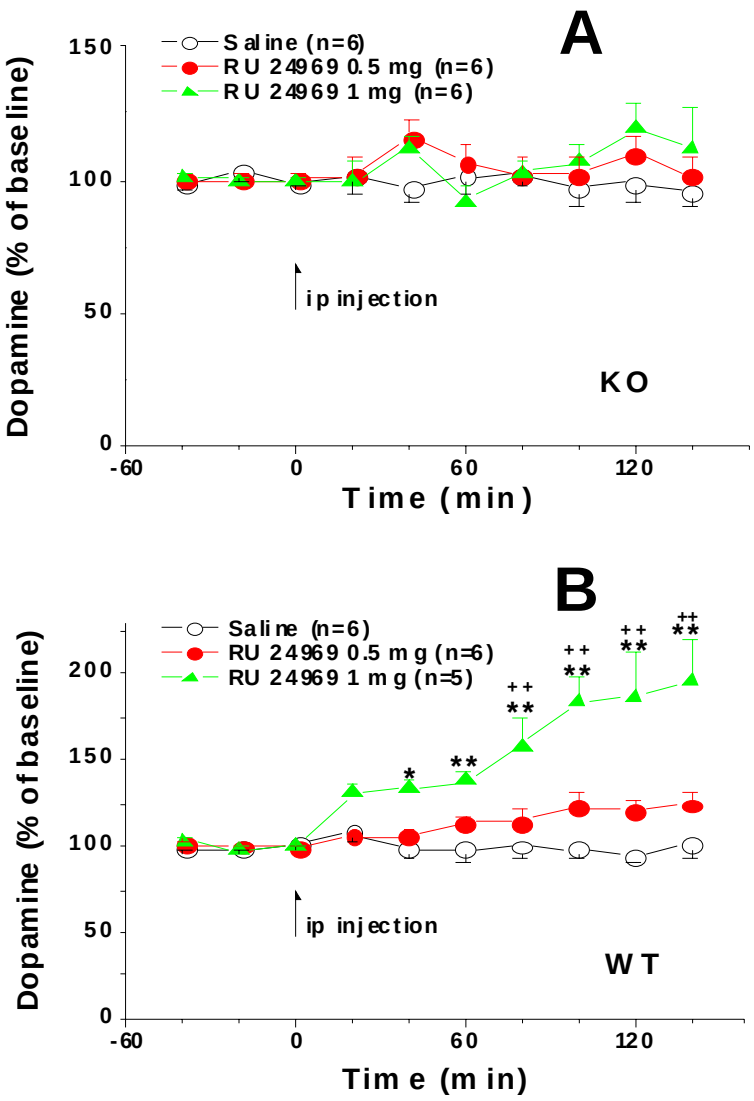

Fig 13. Effects of RU 24969 on extracellular DA concentrations in the NACC of KO (Panel A) and WT (Panel B) mice. RU 24969 (0.5 or 1 mg/kg) or saline was injected ip as indicated by the arrow. Results, expressed as the percentage of baseline values, are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ as compared with the saline group; $^{++}$ $P < 0.01$ as compared with the RU 24969 0.5 mg group (two-way ANOVA followed by Tukey's tests). The basal DA levels (fmol/sample) in the NACC were: 17.05 ± 2.99 (the KO saline group, n=6), 14.79 ± 1.9 (the KO RU 24969 0.5 mg group, n=6), 18.62 ± 2.03 (the KO RU 24969 1 mg group, n=6), 16.93 ± 2.53 (the WT saline group, n=6), 16.01 ± 1.62 (the WT RU 24969 0.5 mg group, n=6), and 17.45 ± 2.43 (the WT RU 24969 1 mg group, n=5). There were no statistically significant differences in the basal DA values between the KO and WT mice (P=0.972), nor were there differences among the saline, RU 24969 0.5 mg, and RU 24969 1 mg groups in the KO (P=0.937) or WT (P=0.423) mice.

9. Effects of pretreatment with SB 216641 or WAY 100635 on RU 24969-induced increases of NACC DA in the WT mice.

Since RU 24969 is a 5-$HT_{1B/1A}$ receptor agonist, it was necessary to assess a possible role of 5-HT_{1A} receptors in the observed effect of 1 mg/kg of RU 24969 on NACC DA in the WT mice. To this end, WAY 100635 (0.1 mg/kg) and SB 216641 (0.1 mg/kg) were used. The dose (0.1 mg/kg) of WAY 100635 was chosen as this dose was able to completely block the effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT but did not cause intrinsic changes in extracellular 5-HT when given alone[17]. The dose (0.1 mg/kg) of SB 216641 was selected based on the facts that the effective receptor antagonist concentration of SB 216641 is similar to that of WAY 100635[25] and that the affinity of SB 216641 (pK_i = 9.0) for the 5-HT_{1B} receptor is almost one order higher than that of RU 24969 (pK_i = 8.2)[7].

In this experiment SB 216641 (ip), WAY 100635 (sc) or saline (the same volume) was administered ip 20 min before RU 24969 (1 mg/kg, ip). In a pilot study using separate groups of WT mice, it was found that administration of SB 216641 at 0.1 mg/kg or WAY 100635 at 0.1 mg/kg did not significantly alter NACC DA in the WT mice for as long as 2 h (data not shown).

As can be seen from Panels A and B of Fig 14, the patterns of the time course of extracellular DA following RU 24969 were similar between the groups of saline plus RU 24969 (Panel A) and WAY 100635 plus RU 24969 (Panel B). Statistical analyses showed that there were no significant differences in extracellular DA concentrations after RU 24969 between these two groups (Panel D). These data suggest that pretreatment with WAY 100635 did not significantly alter the effects of RU 24969 on NACC DA in the WT mice. In contrast, however, pretreatment with SB 216641 completely blocked the ability of RU 24969 to increase NACC DA in the WT mice since no increases in extracellular DA concentrations were seen after RU 24969 in the SB 216641 plus RU 24969 group (Panel C). Statistical analyses showed that extracellular DA concentrations after RU 24969 were significantly lower in the SB 216641 plus RU 24969 group than those in either the saline plus RU 24969 group or the WAY 100635 plus RU 24969 group (Panel D). These data suggest that pretreatment with SB 216641 antagonizes the effects of RU 24969 on NACC DA in the WT mice.

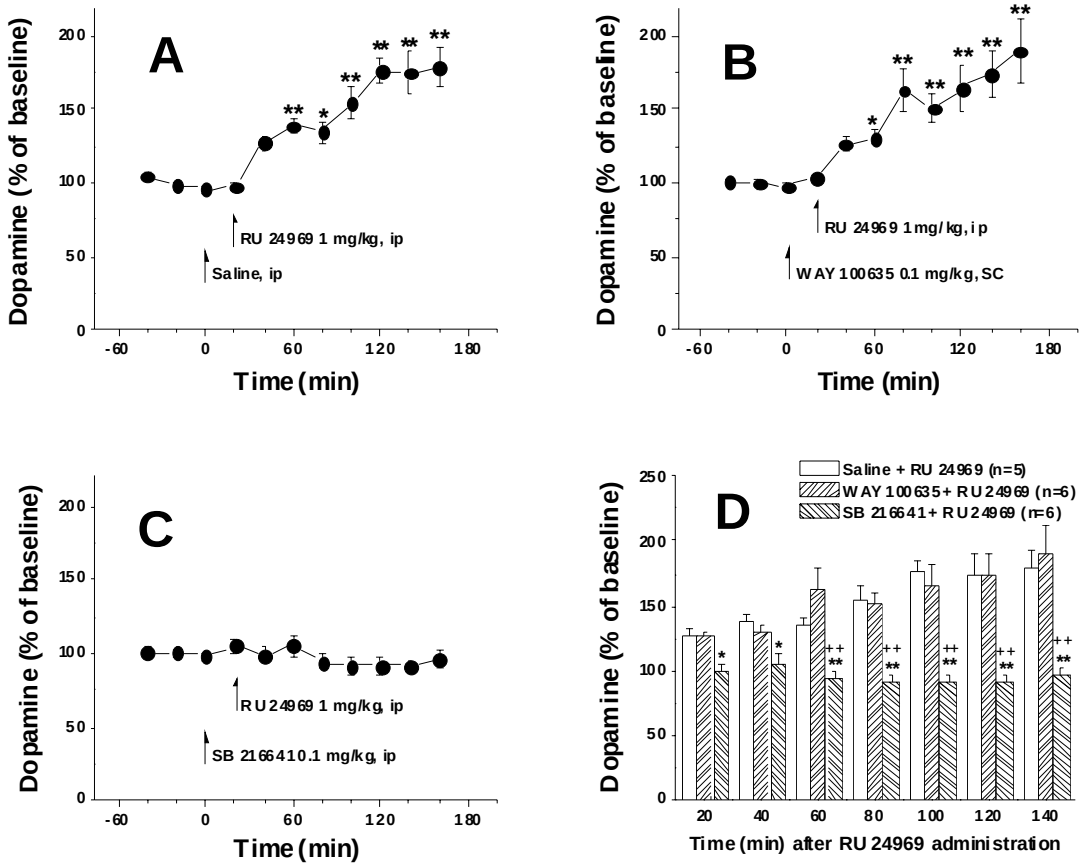

Fig 14. Effects of pretreatment with WAY 100635 or SB 216641 on RU 24969-induced increases of NACC DA in the WT mice. Panels A, B, and C show the time course of NACC DA after pretreatments with saline (Panel A), WAY 100635 (0.1 mg/kg, Panel B), and SB 216641 (0.1 mg/kg, Panel C) followed by 1 mg/kg of RU 24969 twenty minutes later, respectively. * $P < 0.05$, ** $P < 0.01$ as compared with the baseline value (two-way ANOVA followed by Tukey's tests). Panel D shows comparisons of NACC DA after RU 24969 among the groups of saline plus RU 24969, WAY 100635 plus RU 24969, and SB 216641 plus Ru 24969. * $P < 0.05$, ** $P < 0.01$ as compared with the saline plus Ru 24969 group; $^{++}$ $P < 0.01$ as compared with the WAY 100635 plus RU 24969 group (two-way ANOVA followed by Tukey's tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. The basal NACC DA levels (fmol/sample) were: 15.45 ± 2.06 (the saline plus RU 24969 group, n=5), 14.43 ± 1.58 (the SB 216641 plus RU 24969 group, n=6), and 13.69 ± 1.26 (the WAY 100635 plus RU 24969 group, n=6). There were no statistically significant differences in the basal DA values among these groups (P=0.418).

10. **Effects of systemic ethanol on NACC DA in the KO and WT mice.**

After basal DA in the NACC was stable, saline or ethanol at the dose of 1 or 2 g/kg was administered ip to KO or WT mice. As shown in Fig 15, significant increases in NACC DA in the KO mice were observed only after administration of the higher dose (2 g/kg) of ethanol. The lower dose (1 g/kg) of ethanol caused a slight but not statistically significant increase in NACC DA in the KO mice (Panel A). However, administration of the same doses of ethanol all significantly increased NACC DA in the WT mice (Panel B). Comparisons of the NACC DA after 1 g/kg of ethanol show that there are no significant differences in any time points after ethanol between the KO and WT mice (Panel C). However, administration of ethanol at the dose of 2 g/kg produced more pronounced increases of NACC DA in the

WT mice than in the KO mice. Statistical analyses show that significant differences in NACC DA were seen at the time points of 20, 60, 80, and 100 min after ethanol administration between the WT and KO mice (Panel D).

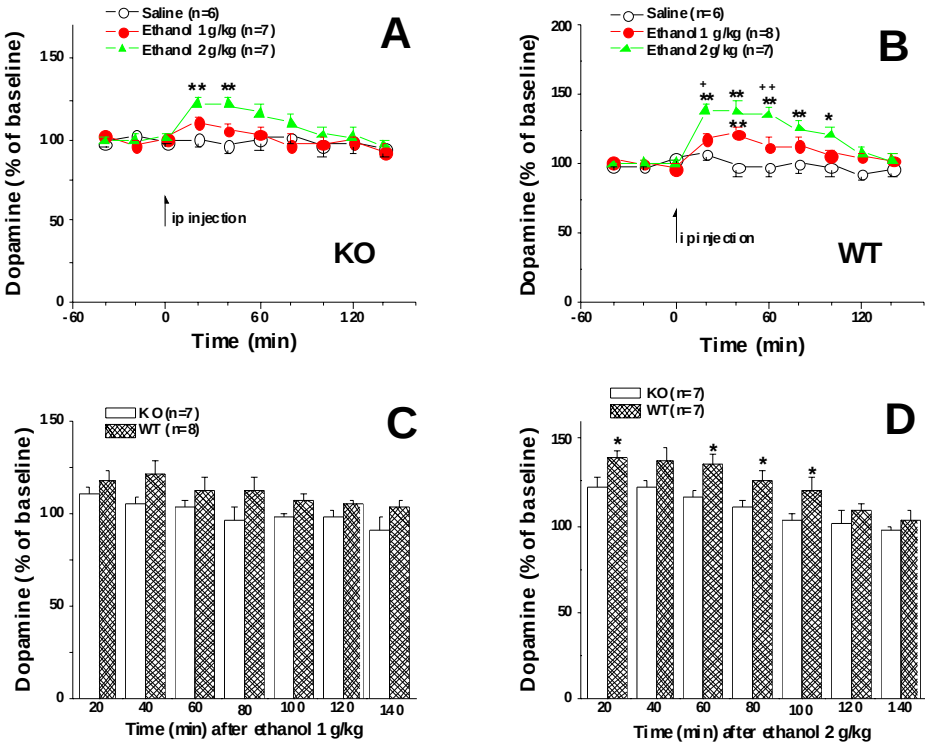

Fig 15. Effects of systemic ethanol on NACC DA in the KO and WT mice. Ethanol (1 or 2 g/kg) or saline was injected ip as indicated by the arrow. Panels A and B show the time courses of extracellular DA in the NACC following saline or ethanol injections in the KO (Panel A) and WT (Panel B) mice, respectively. ***** $P < 0.05$, ****** $P < 0.01$ as compared with the saline group; $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$ as compared with the 1 g/kg ethanol group (two-way ANOVA followed by Tukey tests). Panels C and D show comparisons of NACC DA between the KO and WT mice in response to ethanol at the doses of 1 (Panel C) and 2 g/kg (Panel D), respectively. ***** $P < 0.05$ as compared with the KO mice (two-way ANOVA followed by Tukey tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. The basal DA levels (fmol/sample) were: 13.72 ± 2.28 (the KO saline group, n=6), 15.08 ± 1.65 (the KO ethanol 1 g/kg group, n=7), 12.02 ± 1.12 (the KO ethanol 2 g/kg group, n=7), 13.6 ± 2.11 (the WT saline group, n=6), 14.09 ± 1.47 (the WT ethanol 1 g/kg group, n=8), and 13.25 ± 1.61 (the WT ethanol 2 g/kg group, n=7). There were no statistically significant differences in the basal DA values between the KO and WT mice (P=0.631), nor were there differences among the saline, ethanol 1 g/kg, and ethanol 2 g/kg groups in the KO (P=0.496) or WT (P=0.598) mice.

11. Effects of pretreatment with RU 24969 on ethanol-induced increases of NACC DA in the KO and WT mice.

This experiment compared the effects of the pretreatment with RU 24969 on ethanol-induced increases in NACC DA between the KO and WT mice. After basal DA in the NACC was stable, RU 24969 (0.5 mg/kg) was injected ip to the KO and WT mice, respectively. Twenty minutes later, each mouse received an ethanol injection (1 or 2 g/kg, ip) and extracellular DA in the NACC was monitored.

Fig 16 shows the effects of pretreatment with RU 24969 on the ethanol-induced increase of NACC DA in the KO and WT mice. As shown in Panels A and B, administration of RU 24969 did not

significantly alter the effects of ethanol on NACC DA in the KO mice since extracellular DA concentrations were not significantly different between the ethanol alone group and the ethanol plus RU 24969 group at each time point after administration of ethanol at the dose of 1 (Panel A) or 2 g/kg (Panel B). In the WT mice, although pretreatment with RU 24969 did not significantly alter the effects of 1 g/kg of ethanol (Panel C), it did enhance the effects of 2 g/kg of ethanol on NACC DA (Panel D). As shown in Panel D, in the absence of RU 24969 extracellular DA in the NACC of the WT mice increased to the maximum level after administration of ethanol, then declined gradually and reached the baseline level at 120 min after ethanol injections. In contrast, however, in the presence of the pretreatment with RU 24969 extracellular DA still remained significantly high levels at 120-140 min after ethanol administration as compared with the ethanol alone group. The results suggested that the pretreatment with RU 24969 significantly prolonged the effects of 2 g/kg of ethanol on NACC DA in the WT mice. This type of enhancement of ethanol's effects by activation of 5-HT_{1B} receptors observed in the WT mice was similar to that (Figs 11 and 12) observed in the above experiments performed in the rats.

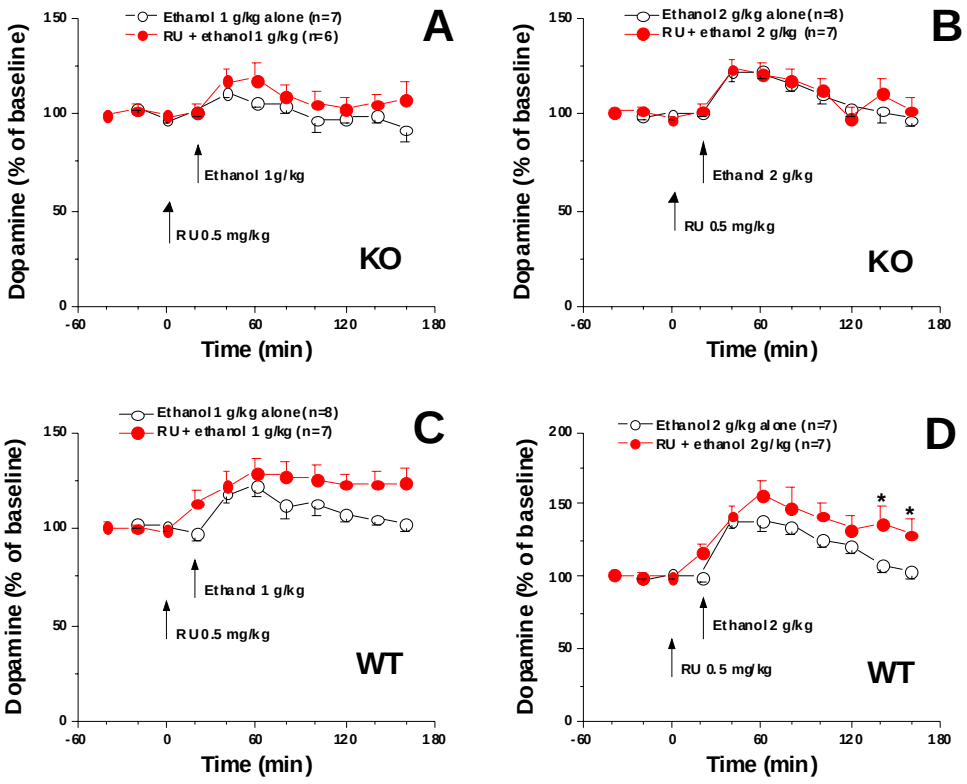

Fig 16. Effects of pretreatment with RU 24969 on ethanol-induced increases of NACC DA in the KO (Panels A and B) and WT (Panels C and D) mice. RU 24969 (0.5 mg/kg) was injected ip as indicated by the arrow. Twenty minutes later, ethanol at the dose of 1 or 2 g/kg was administered indicated by the second arrow. The data of the ethanol alone group in each panel were obtained from Fig 15. ***** $P < 0.05$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. The basal NACC DA levels (fmol/sample) in the KO mice were: 11.01 ± 1.44 (the group of RU 24969 + ethanol 1 g/kg, n=7) and 11.88 ± 1.40 (the group of RU 24969 + ethanol 2 g/kg, n=8); in the WT mice were: 10.96 ± 2.34 (the group of RU 24969 + ethanol 1 g/kg, n=7) and 12.46 ± 2.11 (the group of RU 24969 + ethanol 2 g/kg, n=7).

12. Effects of pretreatment with SB 216641 on 2 g/kg of ethanol-induced increases of NACC DA in the KO and WT mice.

This experiment compared the effects of the pretreatment with SB 216641 on ethanol-induced increases in NACC DA between the KO and WT mice. Since administration of ethanol at the dose of 1 g/kg did not produce significant increases of NACC DA in the KO mice (Panel A of Fig 15), only 2 g/kg of ethanol was used.

SB 216641 (0.1 mg/kg) was injected ip to the KO and WT mice, respectively. Twenty minutes later, each mouse received an ethanol injection (2 g/kg, ip) and extracellular DA in the NACC was monitored. Extracellular DA concentrations in the NACC after ethanol in the presence of SB 216641 (this experiment) were compared with the corresponding values obtained in Fig 15.

As shown in Fig 17, pretreatment with SB 216641 did not significantly alter the effects of ethanol (2 g/kg) on NACC DA in the KO mice (the upper panel). However, ethanol (2 g/kg)-induced increases of NACC DA in the WT mice were significantly attenuated by the pretreatment with SB 216641. Comparisons of NACC DA in response to ethanol administration between the SB 216641 plus ethanol group and the ethanol alone group show that extracellular DA was significantly lower in the former than in the latter at the time points of 20 and 60 min after ethanol injections.

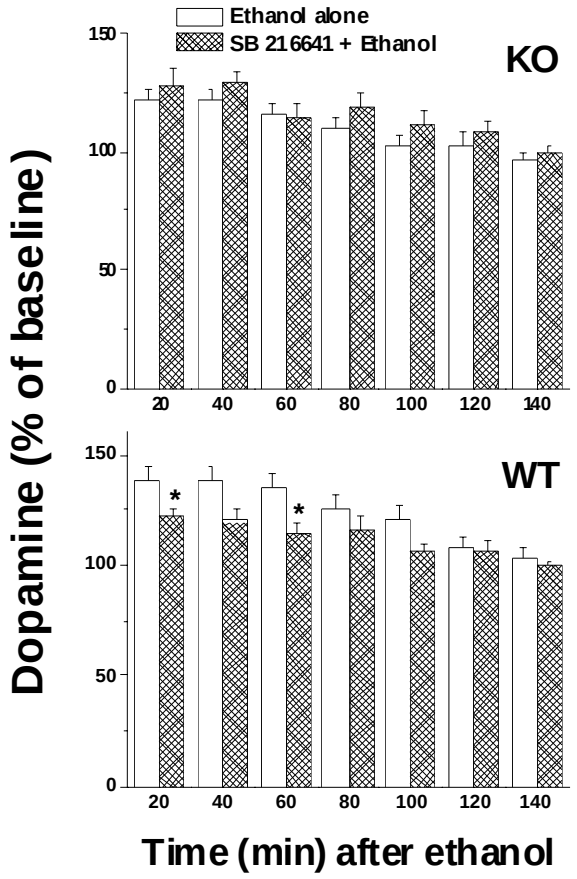

Fig 17. Effects of pretreatment with SB 216641 on ethanol-induced increases of NACC DA in the KO (the upper panel) and WT (the lower panel) mice. SB 216641 (0.1 mg/kg) was injected ip to KO or WT mice. Twenty minutes later, each animal received ip injections of 2 g/kg of ethanol. Extracellular DA concentrations in the NACC after ethanol were compared between the groups of ethanol alone and ethanol plus SB 216641 in the KO (the upper panel) and WT (the lower panel) mice. The data of the ethanol alone group were obtained from Fig 15. ***** $P < 0.05$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. The basal NACC DA levels (fmol/sample) in these two groups were: 13.96 ± 2.11 (KO mice, n=6) and 12.82 ± 1.96 (WT mice, n=6). There were no significant differences in basal DA levels between the KO and WT mice (P=0.414).

The data presented here show that administration of RU 24969 at the dose of 1 mg/kg increases NACC DA in WT mice but not in KO mice and that the RU 24969-evoked increase in NACC DA in the WT mice was completely blocked by SB 216641 but not by WAY 100635, suggesting that activation of 5-HT_{1B} receptors is associated with the RU 24969-evoked increase in NACC DA in the WT mice. Taken

together, the results suggest that RU 24969 may increase NACC DA via the drug-induced activation of 5-HT_{1B} receptors in the WT mice but not the KO mice and support the Hypothesis 1 under the Specific Aim 2.

The present study also shows that systemic administration of ethanol at the dose of 2 g/kg produced more pronounced augmentations of NACC DA in WT mice than in KO mice. The results are consistent with the Hypothesis 2 that was set under this specific aim. These differences in NACC DA following ethanol between the WT and KO mice may not be due to genetic differences in pharmacokinetic processes of ethanol since it has been reported that the KO and WT mice do not differ in ethanol elimination after ip injections[8]. These results, together with the data showing that the RU 24969-induced enhancement and SB 216641-induced attenuation of ethanol's effects occurred only in the WT but not in the KO mice, support the suggestion that activation of 5-HT_{1B} receptors may be involved, at least in part, in the stimulatory effects of ethanol on mesolimbic DA transmission.

In summary, using transgenic animals, the present study provides additional evidence suggesting that activation of 5-HT_{1B} receptors may increase mesolimbic DA transmission and enhance ethanol-induced increases in NACC DA. All these data have resulted in a manuscript (see Appendix 3) that will be submitted to *Brain Research* for publication.

Conclusions: The results support the following hypotheses that were set under the Specific Aim 2: (1) facilitation of NACC DA transmission by activation of the 5-HT_{1B} receptor is absent in 5-HT_{1B} receptor KO mice; (2) systemic ethanol-induced increases in NACC DA is more pronounced in WT mice than in KO mice; and (3) potentiation of the effects of ethanol on NACC DA transmission by activation of the 5-HT_{1B} receptor is absent in 5-HT_{1B} receptor KO mice. These results obtained in transgenic mice are consistent with those obtained in the microdialysis studies using the classic pharmacological technique, and further suggest that activation of 5-HT_{1B} receptors is related to the enhancement of not only mesolimbic DA transmission but also ethanol-induced increases in NACC DA levels.

Specific Aim 3: To determine the involvement of 5-HT_{1B} heteroreceptors on GABA terminals in the VTA in the modulation of DA and GABA release in the VTA, and its involvement in the effect of ethanol in superfused VTA slices.

There were two hypotheses under Specific Aim 3: (1) ethanol increases [^{3}H]DA release from superfused VTA slices via activation of local 5-HT_{1B} receptors and (2) ethanol decreases [^{3}H]GABA release from superfused VTA slices via activation of local 5-HT_{1B} receptors.

13. Effects of ethanol on [^{3}H]DA release from VTA slices.

The rats were killed by decapitation. The brain was removed rapidly from the skull and placed ventral side up on an ice-cold plate. The tissue block containing the VTA was dissected out in a dissecting cryostat (0-4^{0}C) according to the rat brain atlas of Paxinos and Watson (1998), immediately immersed in ice-cold superfusion medium for 3-5 min and chopped into 400 microns slices with a McIlwain tissue chopper under temperature of 0-4^{0}C.

The slices were preincubated at 37^{0}C in 4 ml of superfusion medium (pH=7.4) saturated with 95% O_2/5% CO_2 in the presence of 10 μM pargyline, 0.5 μM desipramine, and 1 μM fluoxetine. After a 15-min preincubation, [^{3}H]DA (final concentration 0.041 μM) was added and the incubation was continued for 30 min in the dark. The slices were then transferred to 12 low-volume (200 μl) chambers of a Brandel superfusion apparatus. Three to four slices were placed between nylon mesh filter discs within each chamber, and superfused at a rate of 1 ml/min at 37^{0}C. The superfusion medium was composed of the following (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 1.3; $MgCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11.1;

Na_2EDTA, 0.004, and ascorbic acid 0.3 (pH 7.4) and continuously aerated with 95% O_2/5% CO_2. After 60-min pre-superfusion, the effluent was collected at 5-min intervals. To examine the effects of ethanol on [^{3}H]DA release, the slices were exposed to the medium containing 0, 25, 50, or 100 mM ethanol for 2 min after the 1-h equilibrium period and sample collection was continued for 30 min after the exposure.

At the end of the experiment, the filter discs with tissue were removed from the chamber and solubilized in 1.25 ml of 1 N NaOH at 37^{0}C for 1 h with gentle agitation. Once the tissue was dissolved, 1.25 ml of 0.8 N HCl was added to neutralize the samples. The tritium contents of the tissue and superfusion samples were measured by liquid scintillation counting at an efficiency of 40%. All experiments were conducted in triplicate. For each 5-min time point, release of [^{3}H]DA was expressed as a fractional release, i.e., as a percentage of the amount of radioactivity in the tissue at the beginning of that collection.

In this experiment the slices were preincubated with low concentrations (0.041 μM) of [^{3}H]DA in the presence of the monoamine oxidase inhibitor pargyline to minimize the formation of DA metabolites. Under these experimental conditions, the majority of tritium collected was shown to be in the form of [^{3}H]DA, not its metabolites[5,6,31].

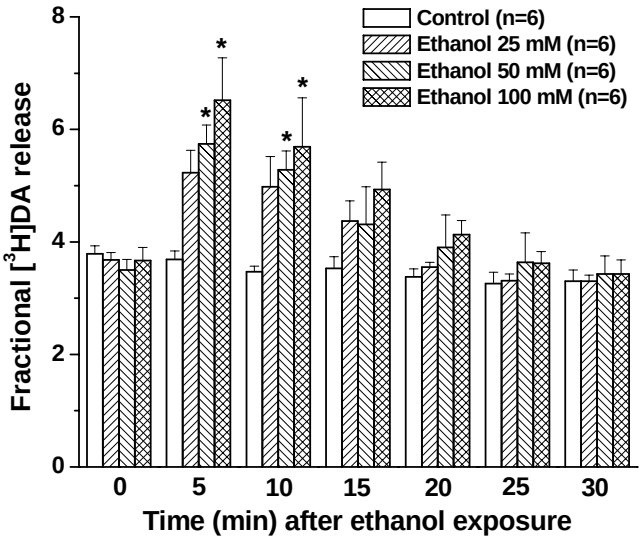

Fig. 18. Effects of ethanol on [^{3}H]DA release from superfused VTA slices. The slices were incubated with [^{3}H]DA and superfused. Ethanol (0, 25, 50, or 100 mM) was added to the perfusion medium for 2 min at 20 min after a 60-min period of equilibrium. Results are mean ± S.E.M. ***** $P < 0.05$ as compared with the control group (no ethanol) (one-way ANOVA followed by Dunnett's test).

As shown in Fig. 18, exposures of the VTA slices to ethanol for 2 min resulted in an immediate increase of [^{3}H]DA release. The maximum increases produced by 25, 50, and 100 mM of ethanol were 142 %, 164%, and 176% of the pre-exposure value, respectively. The effects of 50 and 100 mM of ethanol reached statistical significances as compared with the control group but the effect of 25 mM did not. The [^{3}H]DA release declined after discontinuation of ethanol and reached the pre-exposure value in the third fraction after the ethanol exposure.

The previous microdialysis studies showed that local administration of ethanol through the microdialysis probe into the VTA increased extracellular DA concentrations in this region[32]. Because ethanol can diffuse from the perfusion medium into the tissue through the probe, the increase of DA levels in the VTA detected by microdialysis after local ethanol application could be attributed, at least in part, to the direct actions of ethanol on the tissue within the VTA. Therefore, the data presented here are in good agreement with those obtained from intact animals, and strongly suggest that ethanol may interact directly with the components of the VTA leading to somato-dendritic DA release. Combined with the data

obtained in the above microdialysis experiments showing augmentations of in vivo VTA DA release following systemic ethanol administration (the upper panel of Fig 7, also see Reference 3), the present results support the suggestion that the effects of systemic ethanol on VTA DA release are associated, at least in part, with its direct actions within the VTA.

14. Effects of 5-HT_{1B} receptor agents on the ethanol-induced [^{3}H]DA release from VTA slices.

These experiments were designed to examine the effects of activation or blockade of 5-HT_{1B} receptors on ethanol-induced [^{3}H]DA release from VTA slices. The VTA slices were prepared the same as above and placed in the superfusion chambers. After a 1-h period of equilibrium, the effluent was collected at 5-min intervals. To determine the effects of the 5-HT_{1B} receptor agonist (CP 93129) or antagonist (SB 216641) on the ethanol-induced [^{3}H]DA release, the slices were exposed to ethanol for 2 min twice in each experiment. The first exposure (S1) occurred 20 min after a 60-min equilibrium. It was followed by a second exposure (S2) 50 min later. CP 93129 or SB 216641 was added to the perfusion medium 20 min before the second exposure (S2) and kept throughout the rest of the experiment. Since the first ethanol exposure was carried out in the absence of CP 93129 or SB 216641, it served as an internal control for each set of slices. When SB 216641-CP 93129 interactions were studied, the VTA slices were superfused with SB 216641 for 5 min added prior to CP 93129. Fifty mM (50 mM) of ethanol was chosen because this concentration was shown in the above experiments to produce significant increases of [^{3}H]DA release. The concentrations of CP 93129 and SB 216641 used were 0.1, 0.25, and 0.5 μM.

In these experiments, fractional basal [^{3}H]DA release (B1 and B2) was defined as the amount of radioactivity present in one 5-min sample just before the first (S1) and second (S2) ethanol exposure. The ethanol-induced [^{3}H]DA release (S1 and S2) was expressed as the sum of the increased fractional release above the corresponding baseline (B1 and B2) in the two fractions immediately after the start of ethanol exposure. The effect of the 5-HT_{1B} receptor agent on the ethanol's effect was assessed by comparing the ethanol-induced [^{3}H]DA release (S1) in the absence of that agent to the S2 in the presence of that agent. The ratios of B2/B1 and S2/S1 were calculated for control slices and 5-HT_{1B} receptor agent-treated slices in each experiment.

As shown in the top panel of Fig 19, administration of CP 93129 at the concentration of 0.5 μM, but not 0.1 or 0.25 μM, produced a small but statistically significant increase in basal [^{3}H]DA release. Exposure of the slices to 0.5 μM of CP 93129 was associated with an increase in the ratio of B2/B1 from 0.915 ± 0.045 (the control group, n=6) to 1.098 ± 0.042 (n=5, $P = 0.039$). This increase in basal [^{3}H]DA release induced by 0.5 μM CP 93129 was not significantly altered by the pretreatment with 0.5 μM SB 216641 since there were no significant differences in the B2/B1 ratio between the 0.5 μM CP 93129 alone and 0.5 μM CP 93129 plus 0.5 μM SB 216641 groups ($P = 0.92$).

Administration of CP 93129 also had an effect on ethanol-induced [^{3}H]DA release. As can be seen from the bottom panel of Fig 19, exposures to 0.25 and 0.5 μM CP 93129 were associated with increases in the S2/S1 ratios from 0.931 ± 0.044 (the control group, n=6) to 1.576 ± 0.158 (n=5, $P < 0.01$) and to 1.668 ± 0.183 (n=5, $P < 0.01$), respectively. However, in contrast to basal spontaneous [^{3}H]DA release, the CP 93129-induced enhancement of ethanol-evoked [^{3}H]DA release was significantly inhibited by the pretreatment with SB 216641. As shown in this figure, there were significant differences in the S2/S1 ratio between the 0.25 μM CP 93129 alone and the 0.25 μM CP 93129 plus 0.5 μM SB 216641 groups ($P < 0.01$) and between the 0.5 μM CP 93129 alone and the 0.5 μM CP 93129 plus 0.5 μM SB 216641 groups ($P < 0.01$).

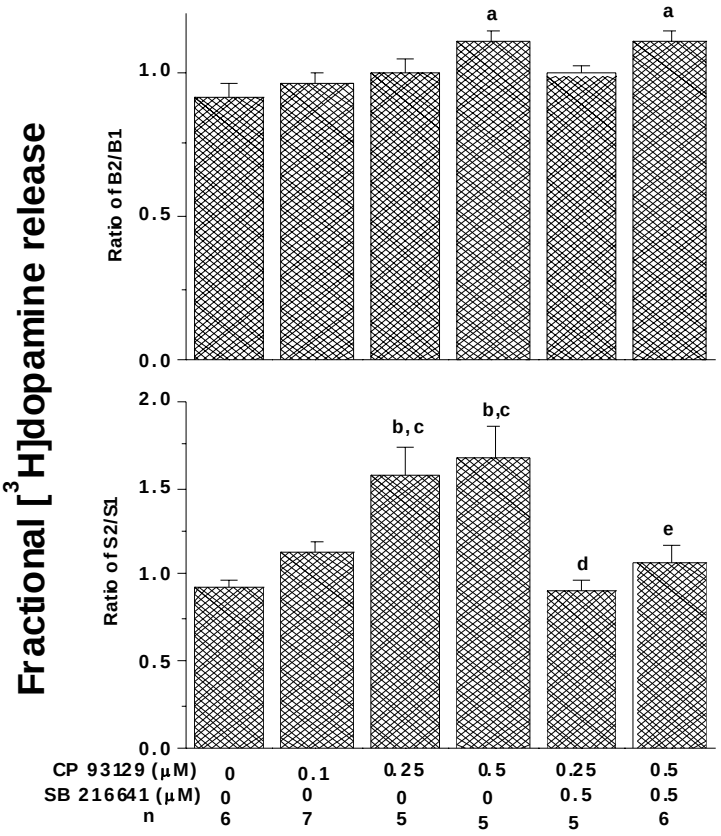

Fig 19. Effects of CP 93129 alone and CP 93129-SB 216641 interactions on basal and ethanol-induced [^{3}H]DA release from superfused VTA slices. The slices were incubated with [^{3}H]DA and superfused, and ethanol-induced [^{3}H]DA release was evoked by two exposures (S1 and S2) to 50 mM ethanol for 2 min. The first exposure (S1) occurred 20 min after a 1-h period of equilibrium. It was followed by a second exposure (S2) 50 min later. CP 93129 (0.1, 0.25, or 0.5 μM) was added 20 min before the S2 and kept throughout the rest of the experiment. When SB216641-CP 93129 interactions were studied, SB 216641 (0.5 μM) was administered 5 min prior to CP 93129. The B2/B1 (top panel) and S2/S1 (bottom panel) ratios were calculated, respectively. Results are mean ± SEM. [a] $P < 0.05$, [b] $P < 0.01$ as compared with the control group; [c] $P < 0.01$ as compared with the 0.1 μM CP 93129 group; [d] $P < 0.01$ as compared with the 0.25 μM CP 93129 group; [e] $P < 0.01$ compared with the 0.5 μM CP 93129 group (one-way ANOVA followed by Student-Newman-Keuls tests).

These data show that CP 93129 increased basal [^{3}H]DA release and enhanced ethanol-induced [^{3}H]DA release. Next, we tested the effects of SB 216641 alone on basal and ethanol-induced [^{3}H]DA release. As shown in Fig 20, administration of SB 216641 at the concentration of 0.1, 0.25, or 0.5 μM had no significant effects on either basal or ethanol-induced [^{3}H]DA release since there were no significant differences in the B2/B1 (the top panel) or S2/S1 (the bottom panel) ratio among the groups.

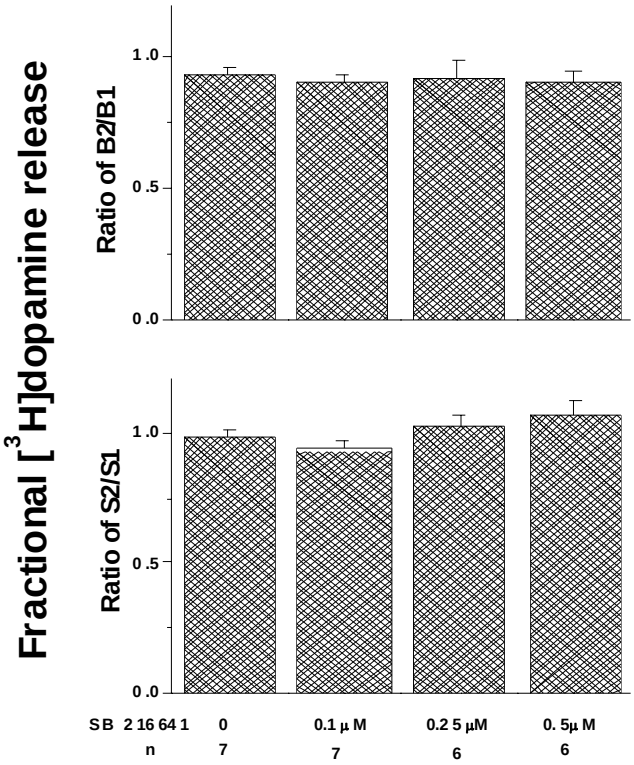

Fig 20. Effects of SB 216641 alone on basal and ethanol-induced [^{3}H]DA release from superfused VTA slices. The slices were incubated with [^{3}H]DA and superfused, and ethanol-induced [^{3}H]DA release was evoked by two exposures (S1 and S2) to 50 mM ethanol for 2 min. The first exposure (S1) occurred 20 min after a 1-h period of equilibrium. It was followed by a second exposure (S2) 50 min later. SB 216641 (0.1, 0.25, or 0.5 μM) was added 20 min before the S2 and kept throughout the rest of the experiment. The B2/B1 (top panel) and S2/S1 (bottom panel) ratios were calculated, respectively. Results are mean ± SEM. There were no statistically significant differences in either the B2/B1 or S2/S1 ratio among the groups (one-way ANOVA).

The data presented here showed that CP 93129 increased basal [^{3}H]DA release. This observation is consistent with the finding by Sarhan et al.[27] who reported that CP 93129 increased basal [^{3}H]DA efflux from rat striatal synaptosomes in a concentration-dependent manner. The present data also showed that administration of CP 93129 enhanced ethanol-induced [^{3}H]DA release from the VTA slices. Interestingly, pretreatment with the 5-HT$_{1B}$ receptor antagonist SB 216641 antagonized the effects of CP 93129 on ethanol-induced [^{3}H]DA release but did not affect the effects of CP 93129 on basal [^{3}H]DA release. These data suggest that CP 93129-induced enhancements of basal and ethanol-induced [^{3}H]DA release may involve different mechanisms. The blockade of CP 93129's effects by SB 216641 suggests that enhancement of ethanol-evoked [^{3}H]DA release by CP 93129 may be associated with the drug-induced activation of VTA 5-HT$_{1B}$ receptors. In contrast, mechanisms rather than the activation of VTA 5-HT$_{1B}$ receptors may be involved in the CP 93129-induced increase in spontaneous overflow of [^{3}H]DA. It has been reported that CP 93129 may induce carrier-mediated DA release[29], which was independent of

activation of 5-HT_{1B} receptors. Whether this mechanism is involved in CP 93129-induced spontaneous overflow of [^{3}H]DA observed in this experiment warrants further investigations..

Using similar superfusion conditions, a previous study carried out in this laboratory showed that administration of CP 93129 at the concentrations of 0.15-0.45 μM, which were similar to those (0.1-0.5 μM) used here, inhibited high potassium-evoked [^{3}H]GABA release from the VTA slices in a concentration-dependent manner and that the CP 93129-induced inhibition of [^{3}H]GABA release was antagonized by pretreatment with SB 216641[33]. These results suggest that activation of 5-HT_{1B} receptors within the VTA may inhibit GABA release in this region. Since VTA DA neurons are under GABAergic inhibitory controls and since the CP 93129-induced inhibition of VTA [^{3}H]GABA release[33] and augmentation of ethanol-induced [^{3}H]DA release were both shown to be mediated through 5-HT_{1B} receptors, it is reasonable to speculate that enhancement of ethanol-induced [^{3}H]DA release by CP 93129 may be a consequence of disinhibition of VTA DA neurons resulting from CP 93129-induced inhibition of VTA GABA release. However, the possibility that the CP 93129-induced increase in basal [^{3}H]DA release via currently unknown mechanisms may also contribute to the drug-induced enhancement of ethanol's effects cannot be ruled out. That is, both a 5-HT_{1B} receptor-mediated and a non-5-HT_{1B} receptor-mediated mechanisms may be involved in the CP 93129-induced enhancement of ethanol-evoked [^{3}H]DA release. Taken together with the data obtained from the above microdialysis studies showing enhancements of ethanol's effects by CP 94253 (Figs 11 and 12), the results further support the suggestion that activation of 5-HT_{1B} receptors within the VTA may enhance the stimulatory effects of ethanol on mesolimbic DA neurons.

In contrast to the observation obtained with CP 93129, the data presented here show that administration of SB 216641 alone did not significantly affect either basal or ethanol-induced [^{3}H]DA release. The inability of SB 216641 to modify basal [^{3}H]DA release suggests that 5-HT_{1B} receptors may not be tonically involved in spontaneous [^{3}H]DA overflow from the VTA slices. This finding is consistent with the previous studies carried out in this and other laboratories showing that spontaneous [^{3}H]DA overflow from the NACC slices[31], from striatal synaptosomes[27], or spontaneous [^{3}H]GABA overflow from the VTA slices[33] is a non-receptor-mediated process. The lack of the effect of SB 216641 on ethanol-induced [^{3}H]DA release suggest that activation of VTA 5-HT_{1B} receptors by ethanol may not be critically involved in the ethanol-induced [^{3}H]DA release and/or that ethanol may not cause sufficient activation of 5-HT_{1B} receptors under the present experimental conditions. This finding is not inconsistent with that obtained with CP 93129. In the case with CP 93129, the drug-induced activation of VTA 5-HT_{1B} receptors may be high enough to inhibit GABA release resulting in disinhibition of mesolimbic DA neurons since it is a potent 5-HT_{1B} receptor agonist. This CP 93129-induced disinhibition and ethanol-induced direct activation of VTA DA neurons could be additive to cause the enhancement of ethanol's effects.

From a superficial point of view, the lack of the effects of SB 216641 on ethanol-induced [^{3}H]DA release is somewhat inconsistent with the previous microdialysis studies showing that infusion of SB 216641 into the VTA significantly attenuated systemic ethanol-induced increases in extracellular DA concentrations in both the VTA and the ipsilateral NACC (Figs 9 and 10). It should be pointed out that the mechanisms underlying ethanol-induced [^{3}H]DA release from tritium-preloaded tissue slices may be different from those underlying systemic ethanol-induced in vivo DA release. For example, it has been suggested that a carrier-mediated mechanism may be involved in ethanol-induced [^{3}H]DA release from superfused NACC slices since it was tetrodotoxin-insensitive, Ca^{2+}-independent, and inhibited by the DA transporter blocker nomifensine[31]. In contrast, however, systemic ethanol-induced DA release in the NACC, detected by microdialysis, appears to be an exocytotic process since it results from ethanol-induced stimulation of the firing activity of mesolimbic DA neurons[12]. It is possible that activation of VTA 5-HT_{1B} receptors may not be critically involved in ethanol-induced [^{3}H]DA release from the VTA in vitro but may indirectly contribute in part to the stimulatory effects of systemic ethanol on VTA DA

neurons in vivo. This may explain why administration of SB 216641 did not modify ethanol's effects observed here but did so in the previous microdialysis experiments (Figs 9 and 10).

15. Effects of ethanol on [^{3}H]GABA release from VTA slices.

The VTA slices were prepared as described above, and preincubated in the presence of 1 mM β-alanine, an inhibitor of the high-affinity GABA uptake into glial cells. After a 15-min preincubation, [^{3}H]GABA (final concentration 0.055 μM) was added and the incubation was continued for 30 min. The slices were then transferred to superfusion chambers and superfused at a rate of 1 ml/min. The superfusion medium was composed of the following (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 1.3; $MgCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11.1; Na_2EDTA, 0.004, nipecotic acid (an inhibitor of the high-affinity GABA transporter system in nerve terminals and glial cells), 0.1; aminooxyacetic acid (an inhibitor of 4-aminobutyrate/2-oxoglutarate aminotransferase which has a major role in GABA degradation), 0.1; and ascorbic acid 0.3 (pH 7.4). To examine the effects of ethanol on [^{3}H]GABA release, the slices were exposed to the medium containing 0, 25, 50, or 100 mM ethanol for 2 min after the 1-h equilibrium period and sample collection was continued for 30 min after the exposure.

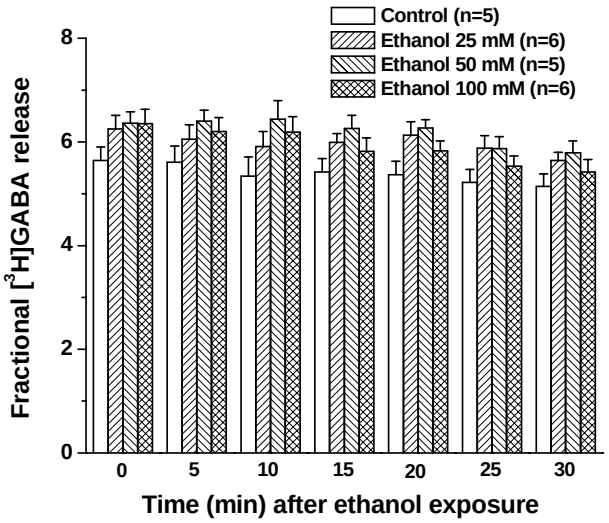

Fig. 21. Effects of ethanol on [^{3}H]GABA release from superfused VTA slices. The VTA slices were incubated with [^{3}H]GABA and superfused. Ethanol (0, 25, 50, or 100 mM) was added to the perfusion medium for 2 min at 20 min after a 60-min period of equilibrium. Results are mean ± S.E.M. No significant differences were detected among the groups at each time point (one-way ANOVA).

As shown in Fig 21, exposure to ethanol did not significantly alter spontaneous [^{3}H]GABA release from the superfused VTA slices. Next, we examined the potential effects of ethanol on electrically induced [^{3}H]GABA release. After a 1-h period of equilibrium, the effluent was collected at 5-min intervals. To determine the effects of ethanol on electrically induced [^{3}H]GABA release, the slices were exposed to electrical field stimulation (120 bipolar pulse, 2-ms long, 20 mA, 0.5 Hz) twice using a Brandel constant current stimulator. The first electrical stimulation (S1) occurred 20 min after a 60-min equilibrium. It was followed by a second electrical stimulation (S2) 50 min later. Ethanol (0, 25, 50, or 100 mM) was added to the perfusion medium 20 min before the second stimulation (S2) and kept throughout the rest of the experiment.

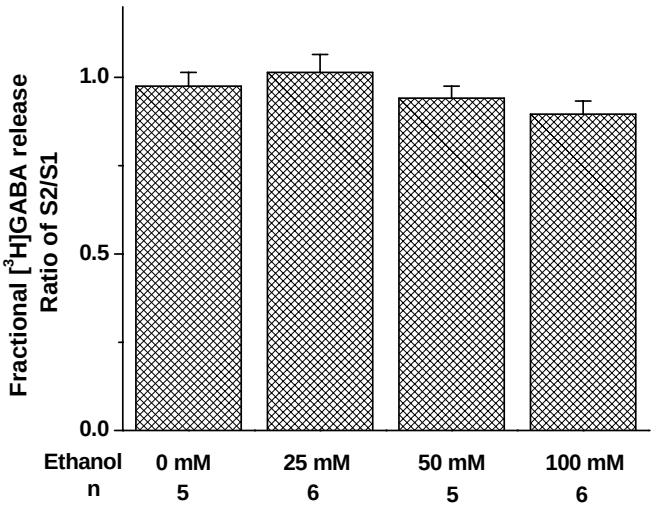

Fig 22. Effects of ethanol on electrical stimulation-induced [^{3}H]GABA release from superfused VTA slices. The slices were incubated with [^{3}H]GABA and superfused, and [^{3}H]GABA release was evoked by two exposures (S1 and S2) to electrical field stimulation. The first stimulation (S1) occurred 20 min after a 1-h equilibrium. It was followed by a second stimulation (S2) 50 min later. Ethanol (0, 25, 50, or 100 mM) was added 20 min before the S2 and kept throughout the rest of the experiment. The S2/S1 ratio was calculated. Results are mean ± SEM. There were no statistically significant differences among the groups (one-way ANOVA).

As can be seen from Fig 22, administration of ethanol had no significant effects on electrical stimulation-induced [^{3}H]GABA release, either, because there were no significant differences in the S2/S1 ratio among the groups of control and treated with different concentrations of ethanol.

Taken together, the present data show that ethanol, at the concentrations that were effective in elevating [^{3}H]DA release, did not significantly modify basal or electrical stimulation-evoked [^{3}H]GABA release from the VTA slices. These data do not support the concept that ethanol may have direct actions on GABA release in the VTA. Together with the data obtained from the previous microdialysis studies showing that ip injections of ethanol at the dose of 1 or 2 g/kg did not significantly alter extracellular GABA in the VTA (Fig 8) but did increased DA concentrations in both the VTA and NACC (Fig 7), the results suggest that acute ethanol may not affect GABAergic transmission in the VTA and do not support the hypothesis that GABA transmission in the VTA may contribute to the stimulatory effect of ethanol on VTA DA neurons.

In summary, ethanol increased [^{3}H]DA release from superfused, tritium-preloaded VTA slices, suggesting that ethanol may directly interact with the VTA causing somato-dendritic DA release. Since no significant changes in either basal or electrical stimulation-induced [^{3}H]GABA release were detected following the same concentrations of ethanol, a GABA mechanism may not be involved in the observed ethanol's effect. The present data also show that CP 93129 enhanced ethanol-induced [^{3}H]DA release and that this effect of CP 93129 was antagonized by SB 216641. This finding is consistent with those obtained from the microdialysis studies and further supports the concept that activation of 5-HT$_{1B}$ receptors enhances ethanol's effects on mesolimbic DA neurons. This 5-HT$_{1B}$ receptor activation-induced enhancement may be mediated, at least in part, through a GABA mechanism since the pervious data show that activation of VTA 5-HT$_{1B}$ receptors inhibits VTA GABA release in vitro[33] and in vivo (Fig 3).

Conclusions: The in vitro results do not support the following hypotheses that were set under the specific aim 3: ethanol increases [^{3}H]DA release and decreases [^{3}H]GABA release from superfused VTA slices via activation of local 5-HT$_{1B}$ receptors. Instead, they suggest that ethanol may not have direct actions on VTA GABA release and that activation of VTA 5-HT$_{1B}$ receptors may not be involved in ethanol-induced [^{3}H]DA release, but may enhance ethanol-induced [^{3}H]DA release probably by additive effects of disinhibition of VTA DA neurons resulting from the receptor activation-induced inhibition of VTA GABA transmission.

KEY RESEARCH ACCOMPLISHMENTS

1. A dual-probe microdialysis, a technically very demanding method, has been established in the PI's lab.
2. The method of genotyping of 5-HT_{1B} receptor KO mice and their counterparts WT mice and the related techniques such as DNA extraction, the polymerase chain reaction (PCR) have been developed in the PI's lab. These molecular techniques, although basic, were new to the PI and the establishment of these methods in the PI's lab is important for our future research.
3. We have had the following findings:
    (1) Activation of VTA 5-HT_{1B} receptors by focal application of the 5-HT_{1B} receptor agonist CP 93129 increased DA concentrations in this region and in the ipsilateral NACC, suggesting the stimulation of mesolimbic DA neurons. The increases of DA release in both regions were antagonized by intra-tegmental infusion of the 5-HT_{1B} receptor antagonist SB 216641 but not the 5-HT_{1A} (WAY 100635) or the 5-HT_{1D} (BRL 15572) receptor antagonist, suggesting the involvement of 5-HT_{1B} receptors. Administration of CP 93129 into the VTA also decreased GABA concentrations in this region, an effect that was also antagonized by SB 216641 but not by WAY 100635 or BRL 15572. However, this effect of CP 93129 on VTA GABA was observed only when a higher concentration was used, suggesting that, in addition to a GABAergic one, other mechanisms may also be involved in CP 93129-induced augmentation of mesolimbic DA transmission.
    (2) Systemic administration of ethanol increased DA concentrations not only in the VTA but also in the ipsilateral NACC. The time course of extracellular DA in the VTA and in the ipsilateral NACC after ethanol was temporally correlated with each other. Moreover, intra-tegmental administration of SB 216641, but not WAY 100635 or BRL 15572, significantly attenuated ethanol-evoked DA release in this region and in the ipsilateral NACC, suggesting that activation of VTA 5-HT_{1B} receptors may be involved in part in mediating the stimulatory effects of ethanol on mesolimbic DA neurons. The underlying mechanism, however, remains to be elucidated, but the present study does not support the involvement of VTA GABA since administration of ethanol at the same doses does not produce significant changes in VTA GABA levels. Furthermore, co-administration of the 5-HT_{1B} receptor agonist CP 94253 into the VTA significantly prolonged the effects of ethanol on extracellular DA concentrations in the ipsilateral NACC, suggesting that further activation of VTA 5-HT_{1B} receptors by the 5-HT_{1B} receptor agonist may enhance the ethanol-induced increase in mesolimbic DA transmission.
    (3) Systemic administration of RU 24969, a 5-$HT_{1B/1A}$ receptor agonist, increased extracellular DA concentrations in the NACC in the WT mice but not in the KO mice. This effect of RU 24969 in the WT mice was antagonized by SB 216641 but not by WAY 100635, suggesting the involvement of 5-HT_{1B} receptors in the effect of RU 24969. Moreover, systemic ethanol produced more pronounced increases of NACC DA in the WT mice than in the KO mice. In addition, the effect of ethanol on NACC DA was enhanced by RU 24969, and attenuated by SB 216641 only in the WT mice but not in the KO mice. These results obtained in transgenic animals are consistent with those obtained in the above microdialysis studies using classic pharmacological approaches, and provide additional support to the hypothesis that activation of 5-HT_{1B} receptors makes a contribution to ethanol-induced increases in mesolimbic DA transmission.
    (4) Ethanol, administered in vitro, increased [^{3}H]DA release from superfused VTA slices, suggesting a direct action on the VTA. This effect of ethanol on [^{3}H]DA release was not affected by SB 216641, suggesting that activation of 5-HT_{1B} receptors may not be involved in ethanol-induced [^{3}H]DA release. However, the ethanol-induced [^{3}H]DA

release was enhanced by co-administration of CP 93129, an effect that was blocked by pretreatment with SB 216641 suggesting the involvement of 5-HT_{1B} receptor activation in the effect of CP 93129. The data showing enhancement of ethanol-induced [^{3}H]DA release by CP 93129 are in good agreement with the findings obtained in vivo, and further support the hypothesis that activation of 5-HT_{1B} receptors may enhance the stimulatory effects of ethanol on mesolimbic DA neurons. However, administration of the same concentrations of ethanol did not alter basal or electrical stimulation-evoked [^{3}H]GABA release, suggesting that ethanol may not have direct actions on GABA components within the VTA.

REPORTABLE OUTCOMES

A. Publication

1. Yan QS, Zheng SZ, Yan SE,: Involvement of 5-HT_{1B} reecptors within the ventral tegmental area in regulation of mesolimbic dopaminergic neuronal activity via GABA mechanisms: a study with dual-probe microdialysis. Brain Research, 1021: 82-91, 2004.
2. Yan QS, Zheng SZ, Feng MJ, Yan SE,: Involvement of 5-HT_{1B} receptors within the ventral tegmental area in ethanol-induced increases in mesolimbic dopaminergic transmission. Brain Research, 1060: 126-137, 2005.
3. Yan QS, Yan SE,: Further evidence that 5-hydroxytriptamine$_{1B}$ receptors modulate mesolimbic dopaminergic transmission and ethanol-induced increases in nucleus accumbens dopamine levels: a study with 5-hydroxytriptamine1B knockout mice. Submitted to Brain Research.

B. Meting presentation and Abstracts

1. Yan QS and Zheng SZ: Involvement of 5-HT-1B receptors within the ventral tegmental area in regulation of mesolimbic dopaminergic transmission via GABA mechanisms. Presented at 2003 Annual meeting of the Society for Neuroscience, New Orleans.
2. Yan QS: Involvement of 5-HT-1B receptors in effects of ethanol on mesolimbic dopamine neurotransmission: A microdialysis study. Presented at 2004 Annual meeting of Research Society on Alcoholism, Vancouver.
3. Yan QS: Involvement of 5-HT1B receptors within the ventral tegmental area in regulation of mesolimbic dopaminergic transmission via GABA mechanisms. Presented at 2004 Military Health Research Forum, Puerto Rico.
4. Yan QS: Involvement of 5-HT_{1B} receptors within the ventral tegmental area in ethanol-induced increases in mesolimbic dopaminergic transmission. Presented at 2006 Military Health Research Forum, Puerto Rico and 2006 Annual meeting of the Society for Neuroscience, Atlanta.
5. Yan QS: Further evidence that 5-hydroxytriptamine$_{1B}$ receptors modulate mesolimbic dopaminergic transmission and ethanol-induced increases in nucleus accumbens dopamine levels: a study with 5-hydroxytriptamine1B knockout mice. Will be presented at 2007 Annual meeting of the Society for Neuroscience, San Diego.

C. Funding received based on work supported by this award:

In order to conduct genotyping of KO and WT mice, several molecular methods have been developed, consequently, the PI has acquired experience and knowledge regarding experiments of molecular biology. With these experience and knowledge, the PI has recently obtained NIH funding (Grant Number: 1 R03 AA016541-01).

D. Training:

Two post-doctoral research associates and two research specialists have been trained through conducting the project supported by this award.

CONCLUSION

1. The data derived from the microdialysis studies performed in rats and 5-HT_{1B} receptor KO mice, and those obtained from the in vitro superfusion experiments all support the hypothesis that activation of VTA 5-HT_{1B} receptors facilitates mesolimbic DA transmission and enhances the stimulatory effects of ethanol on VTA DA neurons. Since increased mesolimbic DA transmission has been implicated in ethanol's rewarding effects[15], the present data may explain in part why 5-HT_{1B} receptors are associated with alcohol abuse in both human genetics[16] and in animal models[9,12], and play a role in regulating alcohol voluntary intakes[28,30]. For example, the reported suppression of alcohol intakes by 5-HT_{1B} receptor agonists[28,30] may be due, at least in part, to the 5-HT_{1B} receptor activation-induced enhancement of ethanol's actions on mesolimbic DA neurons, therefore leading to lesser amounts of alcohol being consumed to produce similar central nervous system effects. Interestingly, a recent study by O'Dell and Parsons[23] showed that activation of VTA 5-HT_{1B} receptors also enhanced cocaine-induced increases in NACC DA levels. This modulation of cocaine's neurochemical effects by 5-HT_{1B} receptors is consistent with the behavioral studies showing that 5-HT_{1B} receptors play a role in the regulation of cocaine's reinforcing and discriminative stimulus effects[11,24]. Thus, 5-HT_{1B} receptor agents may have the potential for the use in prevention and/or treatment of alcoholism and cocaine addiction.

2. Enhancements of mesolimbic DA transmission and the effects of ethanol on mesolimbic DA neurons by the 5-HT_{1B} receptor agonists such as CP 93129 may be a consequence of additive effects resulting from indirect disinhibition of VTA DA neurons due to the 5-HT_{1B} receptor activation-induced inhibition of VTA GABA release. This conclusion was drawn because the pervious[33] and present studies carried out in this laboratory showed that CP 93129 inhibited VTA GABA release both in vitro and in vivo, effects that were also blocked by the 5-HT_{1B} receptor antagonists. However, mechanisms other than GABAergic one may also be involved in CP 93129's effects.

3. Systemic ethanol increased mesolimbic DA neuronal activity. This effect is associated at least in part with ethanol's direct actions within the VTA. However, the present data do not support the hypothesis that ethanol may have effects on VTA GABA transmission either directly or indirectly. Therefore, VTA GABA may not be involved in the stimulatory effects of ethanol on mesolimbic DA neurons. Recent studies suggested that nicotinic acetylcholine receptors within the VTA were involved in the activating effects of ethanol on VTA DA neurons[10,18] and that acetylcholine release was modulated by 5-HT_{1B} receptors[26]. Moreover, 5-HT_{1B} receptors also provide an inhibitory modulation of glutamate release[21]. Glutamatergic afferents to the VTA are thought to play a role in regulating the activity of DA neurons in this region[1]. Therefore, further investigations of potential involvement of cholinergic and/or glutamatergic components within the VTA in the stimulatory effects of ethanol on mesolimbic DA neurons are warranted.

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Research report

Involvement of 5-HT_{1B} receptors within the ventral tegmental area in regulation of mesolimbic dopaminergic neuronal activity via GABA mechanisms: a study with dual-probe microdialysis

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Abstract

This study was designed to assess the involvement of 5-HT_{1B} receptors within the ventral tegmental area (VTA) in the regulation of mesolimbic dopaminergic transmission. Dual-probe microdialysis was performed in freely moving adult Sprague–Dawley rats with one probe within the VTA and the other within the ipsilateral nucleus accumbens (NACC). Drugs were administered into the VTA via retrograde dialysis. Dialysates from both the VTA and the NAC were collected for determination of dopamine (DA) and gamma-aminobutyric acid (GABA) by high-performance liquid chromatography with electrochemical detection. Intra-tegmental infusion of CP 93129 (20, 40, and 80 μM), a 5-HT_{1B} receptor agonist, increased extracellular DA concentrations in a concentration-dependent manner not only in the NACC but also in the VTA, indicating increased mesolimbic DA neuron activity. Administration of CP 93129 at 80 μM into the VTA also significantly decreased extracellular GABA concentrations in this region. Co-infusion of the 5-HT_{1B} receptor antagonist SB 216641 (10 μM), but not the 5-HT_{1A} receptor antagonist WAY 100635 (10 μM) or the 5-$HT_{1D/1A}$ receptor antagonist BRL 15572 (10 μM), antagonized not only the effects of intra-tegmental CP 93129 (80 μM) on VTA DA and NAC DA but also on VTA GABA. The results suggest that activation of VTA 5-HT_{1B} receptors increases mesolimbic DA neuron activities. The increased DA neuron activity may be associated, at least in part, with the 5-HT_{1B} receptor-mediated inhibition of VTA GABA release.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Serotonin receptors

Keywords: Ventral tegmental area; Nucleus accumbens; Dopamine; GABA, 5-HT_{1B} receptor; Dual-probe microdialysis

1. Introduction

Anatomical studies of the afferent pathways to the ventral tegmental area (VTA) have indicated that this region receives serotonergic innervations from the dorsal and medial raphe nuclei [27,39]. Consistent with anatomical evidence, previous studies showed the existence of a functional relationship between 5-hydroxytryptamine (serotonin, 5-HT) and mesolimbic dopamine (DA) neurons within the VTA. Thus, direct administration of 5-HT [25] or the 5-HT_{1B} receptor agonist CP 93129 [64] {3-(1,2,5,6-tetrahydro-4-pyridyl)pyrrolo[3,2-*b*]pyrid-5-one} into the VTA has been found to increase extracellular DA concentrations in the ipsilateral nucleus accumbens (NACC) in vivo, suggesting activation of the mesolimbic DA neurons. Using intracellular recording technique, Pessia et al. [47] reported that 5-HT at concentrations of 3–100 μM depolarized VTA DA neurons in vitro.

The VTA contains DA and non-DA neurons [31]. Many of non-DA neurons are thought to be GABAergic, have been shown to contain GABA, the GABA synthetic enzyme glutamic acid decarboxylase, as well as glutamic

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acid decarboxylase mRNA [40,68]. Electrophysiological features of VTA GABA neurons have recently been characterized by Steffensen et al. [53] and Gallegos et al. [22], and found to be easily distinguished from those of VTA DA neurons. These VTA GABA neurons are presumed to modulate mesolimbic DA neuronal activity in a manner similar to the inhibition of nigrostriatal DA neurotransmission by substantia nigra pars reticulata GABA neurons [54].

It has been reported that 5-HT_{1B} receptors are predominantly located on axon terminals [4,51]. In keeping with this localization, 5-HT_{1B} receptors have been shown to regulate neurotransmitter release from nerve terminals. Activation of 5-HT_{1B} autoreceptors on serotonergic terminals resulted in inhibition of 5-HT release in the frontal cortex [19], hippocampus [50], and striatum [1]. Using electrophysiological techniques, Mlinar et al. [37,38] demonstrated that 5-HT_{1B} receptors were responsible for the presynaptic inhibition of neurotransmission in the CA1 region of the hippocampus. It has also been reported that 5-HT_{1B} receptors can function as a heteroreceptor in some brain areas to inhibit releases of glutamate [3], GABA [12,63] or acetylcholine [11]. Interestingly, recent studies showed that, in addition to 5-HT_{1A} receptors, 5-HT release in rat dorsal and median raphe nuclei was also controlled by 5-HT_{1B} autoreceptors [28] although the presence of 5-HT_{1B} receptors in the raphe nuclei has not been unequivocally demonstrated in histochemical studies [2].

There is a moderately high density of 5-HT_{1B} binding sites in the VTA [6]. A large proportion of 5-HT_{1B} receptors in the VTA is probably located on the terminal of GABAergic cells [7]. Using intracellular recording from midbrain DA neurons in a brain slice containing the VTA, Cameron and Williams [8,9] found that activation of 5-HT_{1B} receptors in the VTA by sumatriptan resulted in reductions of the magnitude of GABA-mediated inhibitory post-synaptic potential, suggesting that VTA 5-HT_{1B} receptors may be involved in modulation of GABA input into VTA DA neurons although the potential involvement of 5-HT_{1D} receptors cannot be ruled out. Our previous studies also showed that activation of 5-HT_{1B} receptors inhibited high potassium-evoked [^{3}H]GABA release from the VTA slices, supporting the hypothesis that 5-HT_{1B} receptors within the VTA can function as heteroreceptors to decrease GABA release in this region [63]. However, these studies were all performed in vitro. At present, direct in vivo evidence supporting the involvement of VTA 5-HT_{1B} receptors in the regulation of mesolimbic DA neuronal activities via GABA mechanisms is lacking.

The present study was designed to address this issue by investigating the role of VTA 5-HT_{1B} receptors for the modulation of DA and GABA release in the VTA and DA release in the ipsilateral NACC in the same animals. Towards this aim, dual-probe microdialysis was used with one in the VTA and the other in the ipsilateral NACC. Several serotonergic agents were administered alone or in combination into the VTA via retrograde microdialysis to minimize the effects of the compounds on the structures other than the VTA. The effects of the drugs on extracellular DA and GABA levels in the VTA and DA levels in the NACC were measured simultaneously.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 250–300 g at the time of surgery, were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA). They were housed at 21±3 °C, 40–60% relative humidity and were maintained under 12-h light/12-h dark conditions with ad libitum access to food and water. All animal care and experimentation were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

2.2. Drugs

WAY-100635 maleate {*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-2-pyridinyl-cyclohexanecarboxamide maleate}, BRL 15572 {4-[3-chlorophenyl]-α-[diphenylmethyl]-1-piperazineethanol hydrochloride} were purchased from Sigma (St. Louis, MO, USA). SB 216641 hydrochloride {*N*-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide hydrochloride} was obtained from Tocris (Ellisville, MO, USA). CP 93129 {3-(1,2,5,6-tetrahydro-4-pyridyl)pyrrolo[3,2]pyrid-5-one} was generously provided by Pfizer (Groton, CT, USA). All drugs were dissolved in water and then diluted to desired concentrations with artificial cerebrospinal fluid (ACSF) before administration. Other reagents used were of analytical grade.

2.3. Microdialysis

The animals were prepared for the microdialysis experiments as described in a previous paper [64]. In brief, surgery was conducted on a Kopf stereotaxic instrument under anesthesia with a combination of sodium pentobarbital (35 mg/kg, i.p.) and halothane (5% in oxygen). Dialysis guide cannulae (Harvard Apparatus, S. Natick, MA, USA) were stereotaxically implanted over both the VTA and the ipsilateral NACC and attached to the skull with dental acrylic and machine screws. The coordinates relative to bregma and skull surface were as follows: the VTA: AP −5.2 mm, L 3 mm (at an angle of 14° from the sagittal plane to avoid rupture of the sagittal sinus), DV 8.0 mm and the

NACC: AP 1.7 mm, L 1.0 mm, DV 8.0 mm according to the atlas of Paxinos and Watson [46]. The period of post-surgical recovery was at least 5 days. On the evening of the day before the experiment, each rat was placed in a plexiglas chamber and dialysis probes (1 and 2 mm in length for the VTA and NACC, respectively), made from cellulose acetate hollow fibers (I. D. 215±15 μm, molecular weight cut-off=6000; Spectrum Medical Industries, Los Angeles, CA, USA), were inserted while gently restraining the freely behaving rat. On the experimental day, ACSF, which contained (in mM) Na^+ (150), K^+ (3.0), Ca^{2+} (1.2), Mg^{2+} (0.8), Cl^- (155), was perfused at 2 μl/min. After 3–4 h, dialysate samples from both the VTA and NACC were collected at 20-min intervals into vials containing 5 μl 0.1 N HCl, and stored at −80 °C until analysis. Dialysate samples from the VTA were divided into two portions with one portion for measurements of DA and the other for determinations of GABA. Frozen samples showed no signs of degradation for up to 1 month in our previous studies [61,62,64]. All treatments were administered via a dialysis probe.

In order to evaluate the implantation of the probe functionally, each dual-probe experiment was finished with infusion of 50 μM of baclofen, a $GABA_B$ receptor agonist, into the VTA probe and the response of extracellular DA in the ipsilateral NACC was determined. A significant decrease (≥50% deduction) in extracellular DA in the ipsilateral NACC after perfusion with baclofen was considered an appropriate implantation of the probe.

2.4. Analytical and histological procedure

For measurement of DA, dialysate samples were injected onto a high performance liquid chromatography (HPLC) system with electrochemical detection. This system consisted of an ESA solvent delivery system (model 580), an ESA microbore column (MD-150×1/RP-C18, 3 μM) for the VTA samples or an ESA narrowbore column (MD-150×2/RP-C18, 3 μM) for the NACC samples, and an ESA coulochem II electrochemical detector equipped with a dual electrode analytical cell (Model 5041) and a guard cell (Model 5020). The guard cell was set at 400 mV, electrode at 175–200 mV with respect to palladium reference electrodes. A VICI micro-electric two-position valve actuator with a 5-μl (for the microbore column) or 50-μl (for the narrowbore column) injection loop was used for sample injection. For VTA DA determination, 10-μl dialysate samples were injected to ensure that the 5-μl injection loop was completely filled. For NACC DA assay, 20-μl dialysate samples were injected onto the column. The mobile phase contained 75 mM Na_2HPO_4, 1.53 mM sodium dodecyl sulfate, 25 μM EDTA, 100 μl/l triethylamine, 11.5% acetonitrile and 11.5% methanol (pH 5.6 with H_3PO_4), and was pumped through the system at 0.07 (for the microbore column) or 0.25 (for the narrowbore column) ml/min. Chromatograms were integrated, compared with standards run separately on each experimental day, and analyzed using a computer-based data acquisition system (EZChrom Chromatography Data System, Scientific Software, San Ramon, CA, USA). The detection limit for dopamine was ~4 fmol at a 2:1 signal-to-noise ratio.

For determination of GABA, an isocratic HPLC system with electrochemical detection was used. This system consisted of an ESA solvent delivery system (model 580), an ESA autosampler (Model 542), a Waters Xterra™ MS column (50×3 mm, C18, 2.5 μM), and an ESA coulochem III electrochemical detector equipped with an analytical cell (Model 5011) and a guard cell (Model 5020). The guard cell was set at 650 mV, and the analytical cell at 250 mV (E1) and 550 mV (E2). The mobile phase contained 100 mM Na_2HPO_4, 0.13 mM Na_2EDTA and 28% methanol (pH 6.4 with H_3PO_4), and was pumped through the system at 0.5 ml/min. Pre-column derivatization with *o*-phthaldialdehyde (OPA)/2-mercaptoethanol was performed automatically by the autosampler by mixing 15 μl of the working derivatizing reagent with 20 μl of dialysate samples or working standard solutions for 2 min. The detection limit for GABA was ~50 pg at a 2:1 signal-to-noise ratio.

After completion of the dialysis, the animals were given an intracardiac perfusion with buffered saline and 10% formalin solutions under anesthesia with sodium pentobarbital, and then decapitated. The brains were removed quickly, and 40-μm-thick coronal sections were cut on a freezing microtome, stained with neutral red and analyzed in the light microscope. The heavy staining of gliosis along the guide cannula track permitted reliable location of the deepest point of penetration. A 2-mm-long (in the NACC) or 1-mm-long (in the VTA) dialysis membrane extended below the tip of the guide cannula. The point of the probe tip was then marked on coronal sections from the atlas of Paxinos and Watson [46].

2.5. Data analysis

All values of DA and GABA reported herein represented uncorrected dialysate levels and were expressed as fmol/sample. The volume of the sample for determination of VTA DA, NACC DA, and VTA GABA was 5, 20, and 20 μl, respectively. A two-way analysis of variance (ANOVA) followed by Tukey's tests was applied. All analyses were performed through computer-based software (SigmaStat). The criterion of significance was set at $P<0.05$.

3. Results

Only data from animals with correct probe placements and appropriate accumbal DA responses to perfusion of the VTA with baclofen were included in data analyses. Approximately 75% of the animals that had undergone surgery had both probes correctly implanted in the VTA and NACC, and met the functional criterion.

3.1. Effects of infusion of CP 93129 into the VTA on extracellular DA concentrations in this region (Fig. 1) and in the ipsilateral NACC (Fig. 2)

Three concentrations of CP 93129 (20, 40 and 80 μM) were administered via a probe into the VTA of three groups of rats for 60 min, respectively, and extracellular levels of DA in both regions were monitored simultaneously. In another group of rats (the control group), ACSF was infused into the VTA for the same period as the drug groups and switching between syringes containing ACSF in this group was found to have no significant effects on the dialysate DA levels in the VTA (Fig. 1) or in the ipsilateral NACC (Fig. 2). As shown in Figs. 1 and 2, administration of CP 93129 produced concentration-dependent increases in extracellular DA levels in both the VTA and NACC. In both regions, infusion of 80 μM of CP 93129 caused more pronounced increases than 20 μM (P=0.015 and P=0.045 at 40 and 60 min in Fig. 1 and P=0.046 at 20 min after drug infusion in Fig. 2). The maximum increases of DA levels produced by 20, 40, and 80 μM of CP 93129 were 137%, 182%, and 242% of baseline in the VTA (Fig. 1), and 160%, 181%, 217% of baseline in the NACC (Fig. 2), respectively. The effects of 20 μM of CP 93129 on VTA DA did not reach statistical significance when compared with the control group.

3.2. Effects of infusion of CP 93129 into the VTA on extracellular GABA concentrations in this region (Fig. 3)

As shown in Fig. 3, infusion of ACSF alone (the control group) did not cause significant changes in GABA levels from VTA dialysates. Administration of CP 93129 at the concentration of 20 or 40 μM did not produce significant alterations in the level of VTA GABA either when compared with the control group although there were tendencies towards reductions following drug infusion. However, infusion of CP 93129 at the concentration of 80 μM caused extracellular GABA in the VTA to decrease by 37% of baseline (P=0.043 and P=0.04 at 20 and 60 min after drug application, respectively). Upon comparing the time course of VTA GABA with that of VTA DA or NACC

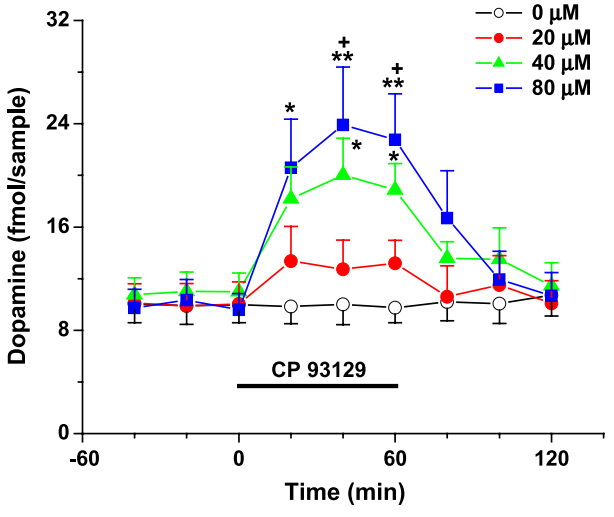

Fig. 1. Effects of local infusion of CP 93129 into the VTA on extracellular DA in this region. CP 93129 (20, 40, and 80 μM) was administered via the probe into the VTA during the period indicated by the bar. Results are mean±S.E.M. from six to seven animals. *P<0.05, **P<0.01 as compared with the control (0 μM) group; +P<0.05 as compared with the 20-μM group (two-way ANOVA followed by Tukey's tests).

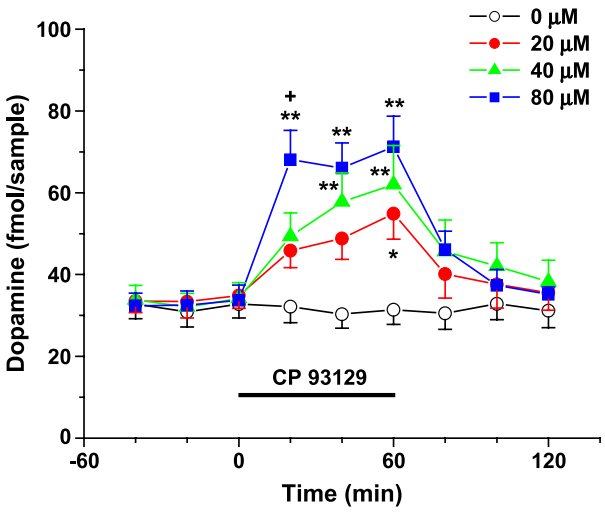

Fig. 2. Effects of local infusion of CP 93129 into the VTA on extracellular DA in the ipsilateral NACC. CP 93129 (20, 40, and 80 μM) was administered via the probe into the VTA during the period indicated by the bar. Extracellular DA in the ipsilateral NACC was monitored by a second probe in this region. Results are mean±S.E.M. from six to seven animals. *P<0.05, **P<0.01 as compared with the control (0 μM) group; +P<0.05 as compared with the 20-μM group (two-way ANOVA followed by Tukey's tests).

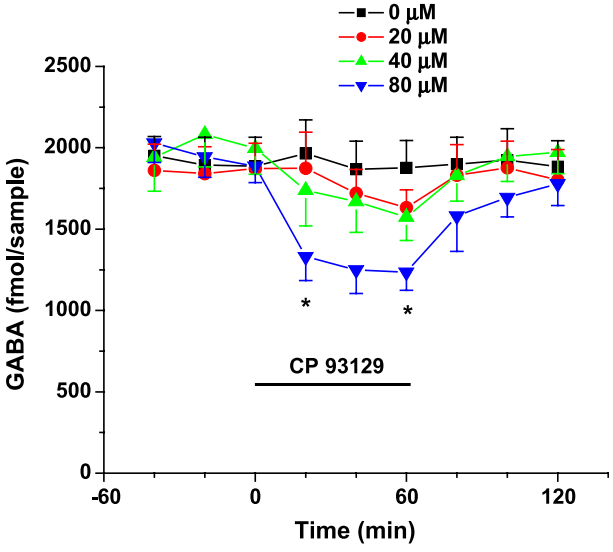

Fig. 3. Effects of local infusion of CP 93129 into the VTA on extracellular GABA in this region. CP 93129 (20, 40, and 80 μM) was administered via the probe into the VTA during the period indicated by the bar. Results are mean±S.E.M. from six to seven animals. *P<0.05 as compared with the control (0 μM) group (two-way ANOVA followed by Tukey's tests).

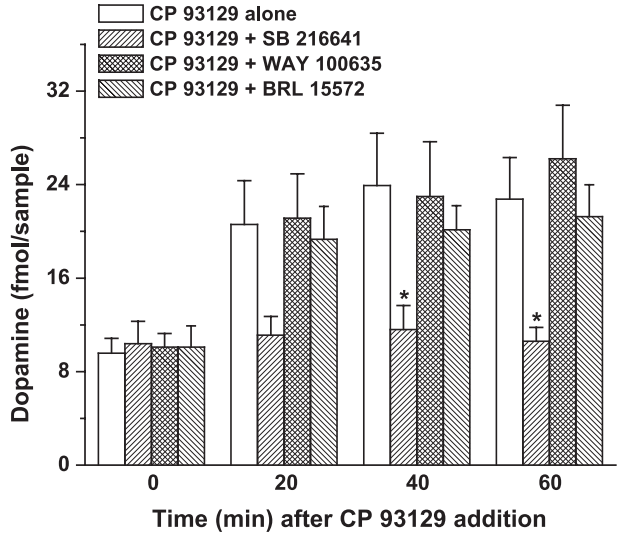

Fig. 4. Comparisons of CP 93129-induced increases of extracellular VTA DA in the presence and absence of WAY-100635, SB 216641, or BRL 15572. WAY-100635 (10 μM), SB 216641 (10 μM), and BRL 15572 (10 μM) were administered through the probe into the VTA for 40 min, and then co-infused with CP 93129 (80 μM) for another 60 min, respectively. Results are mean±S.E.M. from six to seven animals. The data of the group of CP 93129 (80 μM) alone were obtained from Fig. 1. *P<0.05 as compared with the group of CP 93129 alone (two-way ANOVA followed by Tukey's tests).

DA after administration of 80 μM CP 93129, we found that the decrease of VTA GABA was correlated temporarily with the increase of DA in both the VTA and the NACC.

3.3. Effects of WAY-100635, SB 216641, or BRL 15572 on intra-tegmental CP 93129 (80 μM)-induced DA release in the VTA (Fig. 4) and the ipsilateral NACC (Fig. 5)

In these experiments, WAY 100635 (a 5-HT_{1A} receptor antagonist), SB 216641 (a 5-HT_{1B} receptor antagonist), and BRL 15572 (a 5-$HT_{1D/1A}$ receptor antagonist) were used. All these drugs were infused into the VTA at the concentration of 10 μM for 40 min alone and then co-infused with CP 93129 (80 μM) for another 60 min, respectively. The concentration of antagonists was chosen based on the reports in the literature and ~5% of the efficiency of the probe used. It has been shown that 10–100 nM of WAY 100635 potently antagonized the 5-HT_{1A} receptor-mediated effects in the isolated guinea pig ileum [21]. Our previous in vitro study showed that 0.45 μM SB 216641 completely antagonized 5-HT_{1B} receptor-mediated inhibition of [^{3}H]GABA release from rat VTA slices [63]. It has also been reported that the BRL 15572 at the concentration of 0.5 μM blocked the 5-HT_{1D} receptor-mediated effects on in vitro 5-HT release from the rat dorsal raphe nucleus slices [28].

In separate groups of rats infusion of WAY 100635, SB 216641, or BRL 15572 at the concentration of 10 μM into the VTA for 2 h did not significantly alter extracellular DA levels in either the VTA or the ipsilateral NACC (data not shown).

Figs. 4 and 5 show comparisons of intra-tegmental CP 93129 (80 μM)-induced accumbal DA release in the presence and absence of WAY-100635, SB 216641, or BRL 15572. It can be seen from these figures, the CP 93129-induced DA releases in the VTA and the NACC were all significantly attenuated by co-infusion of SB 216641. In the presence of SB 216641, CP 93129 (80 μM)-induced DA outputs in the VTA (Fig. 4) and NACC (Fig. 5) were all significantly lower than those in the absence of SB 216641 (P<0.05, two-way ANOVA followed by Tukey's tests). However, co-administration of WAY-100635 or BRL 15572 had no significant effects on the CP 93129-induced DA release in either the VTA or the NACC.

3.4. Effects of WAY-100635, SB 216641, or BRL 15572 on intra-tegmental CP 93129 (80 μM)-induced reductions of GABA release in the VTA (Fig. 6)

In separate groups of rats infusion of WAY 100635, SB 216641, or BRL 15572 at the same concentration used into the VTA for 2 h did not alter significantly extracellular GABA levels in the VTA (data not shown). As shown in Fig. 6, the basal VTA GABA values did not differ among the experimental groups. Administration of SB 216641, but not WAY 100635 or BRL 15572, antagonized the effects of intra-tegmental CP 93129 on VTA GABA.

At the end of each experiment, baclofen (50 μM) was infused into the VTA. Infusion of baclofen caused extracellular dopamine in the ipsilateral NACC to decrease to 30–

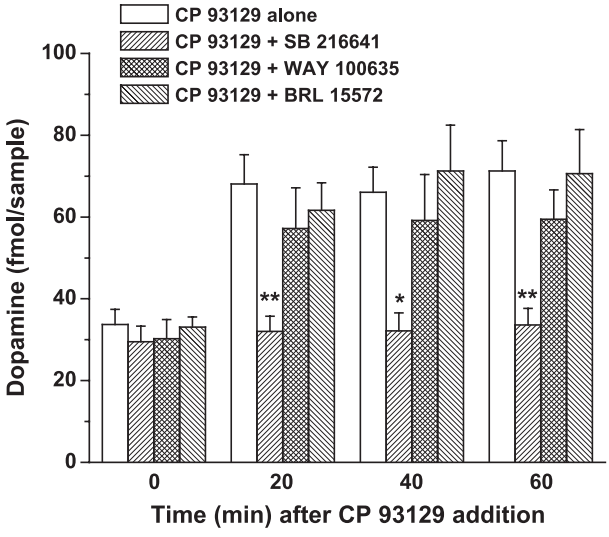

Fig. 5. Comparisons of CP 93129-induced increases of extracellular NACC DA in the presence and absence of WAY-100635, SB 216641, or BRL 15572. WAY-100635 (10 μM), SB 216641 (10 μM), and BRL 15572 (10 μM) were administered through the probe into the VTA for 40 min, and then co-infused with CP 93129 (80 μM) for another 60 min, respectively. Extracellular DA in the ipsilateral NACC was monitored by a second probe in this region. Results are mean±S.E.M. from six to seven animals. The data of the group of CP 93129 (80 μM) alone were obtained from Fig. 2. *P<0.05, **P<0.01 as compared with the group of CP 93129 alone (two-way ANOVA followed by Tukey's tests).

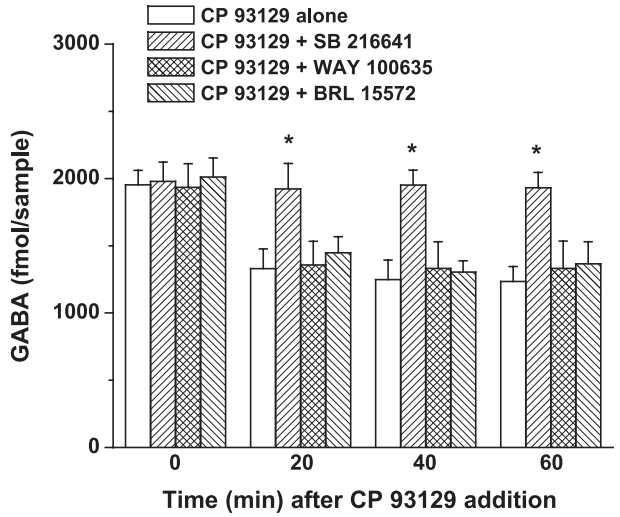

Fig. 6. Comparisons of CP 93129-induced decreases of extracellular VTA GABA in the presence and absence of WAY-100635, SB 216641, or BRL 15572. WAY-100635 (10 μM), SB 216641 (10 μM), and BRL 15572 (10 μM) were administered through the probe into the VTA for 40 min, and then co-infused with CP 93129 (80 μM) for another 60 min, respectively. Results are mean±S.E.M. from six to seven animals. The data of the group of CP 93129 (80 μM) alone were obtained from Fig. 3. *P<0.05 as compared with the group of CP 93129 alone (two-way ANOVA followed by Tukey's tests).

45% of baseline (data not shown). The observed effects of baclofen on NACC DA were consistent with those reported in the literature [57,67], suggesting the functional integrity of the circuitry studied in our dual-probe design.

4. Discussion

The DA release in both somatodendritic (VTA) and axon terminal areas (NACC) were simultaneously determined in this study. Previous studies show that, in addition to classical DA release from synapses in their terminal areas, VTA DA neurons release DA from their somata and dendrites [15,30]. Microdialysis findings have shown that, similar to extracellular DA in the NACC, extracellular DA in the VTA was tetrodotoxin sensitive and calcium dependent [10,13,32], suggesting that a depolarization-induced, exocytosis-mediated, somatodendritic release of DA occurred in the VTA under basal conditions. Evidence also indicated that extracellular DA in the VTA was regulated by D2 autoreceptors and DA reuptake processes [14,32]. Taken together, the data suggest that somatodendritic DA in the VTA, like that released from axon terminals in the NACC, was also dependent on the neuronal firing rate and thus can be used as an index of the activity of mesolimbic DA neurons. Drugs that increase the firing rate of mesolimbic DA neurons increase the extracellular DA levels in both the VTA and the NACC when they are focally applied into the VTA. For example, intra-tegmental administration of nicotine, a drug that increases mesolimbic DA transmission by augmentations of DA neuronal firing rates [36,41], increased both somatodendritic DA release in the VTA and synaptic DA release in the NACC [66]. However, administration of cocaine into the VTA has been found to decrease NACC DA while increasing VTA DA [16], a finding that was consistent with inhibitory effects of cocaine on DA cell firing mediated by the drug-induced increase of VTA DA. Therefore, determination of both somatodendritic and axon terminal DA release may provide more complete profiles of mesolimbic DA neuronal activities.

The present in vivo study shows that local application of CP 93129, a 5-HT_{1B} receptor agonist, into the VTA increased not only somatodendritic DA release in this region but also DA release from nerve terminals in the NACC, consistent with the excitation of mesolimbic DA neurons. CP 93129 is a putatively specific 5-HT_{1B} receptor agonist, with ≥150-fold higher affinity for 5-HT_{1B} vs. other 5-HT_1 and 5-HT_2 ligand binding sites (IC_{50} values: 5-HT_{1A} 3000±400, 5-HT_{1B} 15±5, 5-HT_{1D} 2200±700, and 5-HT_2> 10,000 nM) [17,35]. It is also claimed to lack substantial affinity for dopamine, noradrenaline or opiate receptors [35]. The concentrations of CP 93129 used in this study were 20–80 μM. Considering approximately ~5% of the efficiency of the probe used in the VTA, it is estimated that actual concentrations of CP 93129 in the extracellular fluid immediately adjacent to the dialysis membrane may be in the range of 1–4 μM. This poses the possibility that the selectivity of CP 93129 for the 5-HT_{1B} receptors may have been compromised. Although this possibility cannot be ruled out, two aspects of the dialysis technology may require the high concentrations. First, the rate constant of diffusion of compounds from the perfusion medium into the brain may be high enough that higher concentrations in the perfusate are necessary to maintain concentrations in the neuropil; these concentrations can be achieved at lower levels in in vitro preparations where a significant diffusion barrier is not present. Second, in addition to diffusion across the dialysis membrane, a concentration gradient away from the probe surface into the neuropil will exist. Thus, to achieve pharmacologically active concentrations in the neuropil not adjacent to the dialysis membrane it may be necessary to have higher drug concentrations in the tissue adjacent to the probe.

To assess the involvement of 5-HT_{1B} receptors in CP 93129's actions, WAY-100635 (a 5-HT_{1A} receptor antagonist) [21], SB 216641 (a 5-HT_{1B} receptor antagonist) [26,48], and BRL 15572 (a 5-$HT_{1D/1A}$ receptor antagonist) [26,48] were used. The affinities (given as pK_i) of these antagonists for 5-HT-$_{1A}$,-$_{1B}$, and-$_{1D}$ receptor subtypes are as follows: WAY 100635: 8.9, <6, and <6; SB 216641: 6.3, 9.0, and 7.6; BRL 15572: 7.7, 6.1, and 7.9 [26,48]. All these drugs were tested for their impacts on the actions of CP 93129 at a highest concentration used, i.e., 80 μM, since, theoretically, higher concentrations of CP 93129 would have less selectivity as compared with lower concentrations. If 5-HT_{1A} receptors are involved in the actions of CP 93129, WAY 100635 (pK_i=8.9 for the 5-HT_{1A} receptor) and BRL

15572 (pK_i=7.7 for the 5-HT_{1A} receptor) would to some degree antagonize the effects of CP 93129 on VTA DA or NACC DA. However, this is not the case. The data presented here show that administration of neither WAY 100635 nor BRL 15572 into the VTA antagonizes the effects of intra-tegmental CP 93129 on extracellular DA in either the VTA or the NACC. These results are in opposition with the involvement of 5-HT_{1A} receptors in the observed CP 93129's effects.

Until recently, it has been impossible to distinguish pharmacologically between 5-HT_{1B} and 5-HT_{1D} receptors, but compounds have now been identified which show selectivity for both receptor subtypes. SB 216641 has been reported to have high affinity and selectivity for 5-HT_{1B} over 5-HT_{1D} receptors [48]. This drug shows more than 10-fold higher selectivity at 5-HT_{1B} (pK_i=9.0) receptors compared to 5-HT_{1D} (pK_i=7.6) [48]. BRL 15572 is recently identified as a selective 5-HT_{1D} receptor antagonist. It is more than 60 fold selective for 5-HT_{1D} over 5-HT_{1B} receptors [48]. It has been shown that the hypothermic responses to the 5-$HT_{1B/1D}$ receptor agonist SKF-99101H [3-(2-dimethylaminoethyl)-4-chloro-5-propoxyindole hemifumarate] was dose-dependently blocked by SB 216641 but not by BRL 15572 [26]. It has also been reported that CP 93129 (0.3 μM)-induced inhibition of 5-HT release was antagonized by SB 216641 at concentrations of 0.05 and 0.2 μM but remained unaffected in the presence of BRL 15572 (0.5 μM) [28]. The present data show that intra-tegmental CP 93129-induced augmentations of VTA DA and NACC DA were antagonized by local infusion of 10 μM SB 216641. Considering approximately 5% of the probe efficiency, it could be estimated that the actual concentration of SB 216641 in the extracellular fluid may be in the range of ~0.5 μM. Therefore, inhibitory effects seen with SB 216641 might be afforded by its antagonism either at 1B or 1D or both receptor subtypes since, as mentioned above, the p*Ki* values of the drug for 1B and 1D receptor subtypes are 9.0 and 7.6. However, the results with BRL 15572 do not support the involvement of the 1D subtype in the actions of CP 93129. In the present study, BRL 15572 was administered at the same concentration (10 μM) as that of SB 216641. If antagonism at the 1D subtype makes a contribution to the observed effects of SB 216641, administration of BRL 15572 would also produce antagonistic effects which are similar in magnitude to those by SB 216641 since the former has similar or even higher affinity (pK_i=7.9) for the 1D subtype than does the latter (pK_i=7.6). However, the data presented in this study show that intra-tegmental CP 93129-induced augmentations of VTA DA and NACC DA were significantly antagonized only by local infusion of 10 μM SB 216641 but not by 10 μM BRL 15572. These data are consistent with the involvement of 5-HT_{1B} receptors but not 5-HT_{1D} receptors in CP 93129's actions. Taken together, the present results suggest that increased mesolimbic DA neuron activities following intra-tegmental administration of CP 93129 may be associated with the drug-induced activation of 5-HT_{1B} receptors within the VTA.

As mentioned in Introduction, The VTA contains DA and non-DA neurons [31]. Many of non-DA neurons are thought to be GABAergic, have been shown to contain GABA, the GABA synthetic enzyme glutamic acid decarboxylase, as well as glutamic acid decarboxylase mRNA [40,68]. Neuropharmacological findings indicate that VTA DA neurons appear to receive tonic inhibitory inputs from these GABAergic neurons and from descending GABA-containing terminals originated from the NACC and the globus pallidus [33,59]. Using the reverse transcription-polymerase chain reaction technique, Okada et al. [42] observed that various $GABA_A$ receptor subunits were expressed in VTA DA neurons. The blockade of $GABA_A$ receptors within the VTA with focal application of bicuculline [57] or picrotoxin [29] has been reported to increase extracellular DA concentration in the ipsilateral NACC, suggesting the augmentation of mesolimbic DA neuronal activity.

The present study shows that local infusion of CP 93129 at the concentration of 80 μM caused a decrease of extracellular GABA in the VTA. This effect of CP 93129 was also antagonized by local administration of SB 216641 but not by either WAY 100635 or BRL 15572. The results suggest that it is activation of VTA 5-HT_{1B} receptors that may be associated with the drug-induced reduction of GABA release in this region. These data are in good agreement with our previous in vitro studies showing that activation of 5-HT_{1B} receptors by CP 93129 or RU 24969 inhibited high potassium-evoked [^{3}H]GABA release from rat VTA slices in a concentration-dependent fashion [63]. Chadha et al. [12] also reported that activation of 5-HT_{1B} receptors by CP 93129 (0.6–16.2 μM) produced a concentration-dependent inhibition of 25 mM KCl-evoked [^{3}H]GABA release from slices of the rat globus pallidus. Since VTA DA neurons are under inhibitory GABAergic controls as mentioned above, reductions of VTA GABA release by 80 μM of CP 93129 may account for the drug-induced increase of VTA DA neuronal activity. That is, intra-tegmental infusion of CP 93129 may act at 5-HT_{1B} heteroreceptors to decrease GABA release in the VTA. The reduction of inhibitory GABAergic input to the VTA DA neurons would lead to disinhibition of these neurons, consequently resulting in increases in both somatodendritic and axon terminal DA release.

The present study shows that administration of CP 93129 at lower concentrations (i.e., 20 or 40 μM) did not significantly alter GABA levels in dialysates from the VTA but did increase extracellular DA in the VTA and the NACC. The results suggest that, in addition to indirect disinhibition of VTA DA neurons resulting from decreased GABAergic control, other mechanisms may also be involved in CP 93129-induced augmentations of mesolimbic DA transmission. On the other hand, previous microdialysis studies indicate that the part of GABA as measured

by microdialysis does not fulfill the classical criteria for exocytotic release [55], suggesting that GABA levels monitored by microdialysis probes may derive from non-neuronal pools in addition to the neuronal origin. Therefore, it is possible that decreased exocytotic GABA release resulting from lower concentrations of CP 93129 (i.e., 20 or 40 μM) may only cause a small change in total extracellular GABA levels that cannot be detected by our HPLC system.

We observed that administration of 10 μM SB 216641 alone into the VTA for 2 h did not significantly alter basal GABA concentrations in this region (data not shown). This finding may be reflective of the limits of detection as mentioned above. However, this finding may also suggest that 5-HT_{1B} receptors within the VTA are not involved in the modulation of GABA levels in this region during normal tonic or basal conditions. This speculation is in agreement with the finding that administration of 10 μM SB 216641 alone into the VTA did not significantly alter basal VTA DA or NACC DA either (data not shown).

The interaction between 5-HT and DA via the 5-HT_{1B} receptor within the VTA may play an important role in several behavioral disorders such as drug abuse. 5-HT_{1B} receptors have been shown to enhance the reinforcing properties of both cocaine and the selective dopamine reuptake inhibitor GBR 12909 [43,52]. Recently, Filip et al. [20] showed that intra-tegmental microinjection of the 5-HT_{1B} receptor agonist and antagonist increased and decreased the discriminative stimulus effects of cocaine, respectively, suggesting that the tegmental 5-HT_{1B} receptors were necessary for full expression of cocaine discrimination. Infusion of CP 93129 into the VTA also dose-dependently potentiated cocaine-induced increases of NACC DA efflux and motor activation in cocaine-naïve rats [45]. Studies carried out in mice lacking 5-HT_{1B} receptors indicated that 5-HT_{1B} receptors are important for ethanol's rewarding effects [18,49]. It has been demonstrated that VTA DA neurons play a critical role in the reinforcing/rewarding properties of many drugs of abuse [34]. Since inhibition of GABA release within the VTA may lead to activation of VTA DA neurons by disinhibition, this increased activity of VTA DA neurons may be associated with 5-HT_{1B} receptor-mediated regulation of rewarding effects of abused drugs. In fact, there is a report in the literature showing that the potentiation of cocaine reinforcement by the 5-HT_{1B} receptor agonist was mediated by a potentiation of increases of mesolimbic DA transmission via GABA mechanisms [44]. Investigation of the interaction among 5-HT, DA and GABA within the VTA via the 5-HT_{1B} receptor would provide further insight into mechanisms regulating behavioral responses to abused drugs, thereby, providing for alternative approaches to treatment of drug addiction. For example, ethanol has been reported to stimulate mesolimbic DA neurons [5,23] leading to increased DA release in the VTA [10] and the NACC [60]. Local infusion of ethanol also increased extracellular 5-HT concentrations in the VTA [65]. The investigation of possible involvement of VTA 5-HT_{1B} receptors in the regulation of the effects of ethanol on the mesolimbic DA neuronal activity may explain why 5-HT_{1B} receptors are important for modifying the reinforcing [49], discriminative stimulus effects [24], and voluntary intakes [56,58] of ethanol.

In summary, the present results suggest that activation of VTA 5-HT_{1B} receptors is associated with CP 93129-induced activation of mesolimbic DA neurons. This conclusion is based on the fact that intra-tegmental CP 93129 increased DA release in both the VTA and the NACC and that the increase of the DA release was antagonized by the 5-HT_{1B} receptor antagonist but not by the 5-HT_{1A} or 5-HT_{1D} receptor antagonist. The results also suggest that the 5-HT_{1B} receptor-mediated inhibition of VTA GABA release may contribute to the observed activation of mesolimbic DA neurons. This conclusion is drawn from the fact that intra-tegmental CP 93129 concomitantly caused a reduction of VTA GABA, an effect that was also blocked by the 5-HT_{1B} receptor antagonist. In addition to this indirect disinhibition of VTA DA neurons resulting from decreased GABAergic control, other mechanisms may also be involved in CP 93129-induced augmentation of mesolimbic DA transmission, particularly under lower concentrations of the drug. This speculation is based on the fact that CP 93129 increased DA release at all concentrations tested but significantly decreased VTA GABA release only at a higher concentration.

Acknowledgments

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Research Report

Involvement of 5-HT_{1B} receptors within the ventral tegmental area in ethanol-induced increases in mesolimbic dopaminergic transmission

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Abstract

Evidence suggests that 5-hydroxytriptamine-1B (5-HT_{1B}) receptors play a role in modifying ethanol's reinforcing effects and voluntary intake, and that 5-HT_{1B} receptors within the ventral tegmental area (VTA) are involved in regulation of mesolimbic dopaminergic neuronal activity. Since increased mesolimbic dopaminergic transmission has been implicated in ethanol's reinforcing properties, this study was designed to assess the involvement of VTA 5-HT_{1B} receptors in mediating the stimulatory effects of ethanol on VTA dopaminergic neurons. Dual-probe microdialysis was performed in freely moving adult Sprague–Dawley rats with one probe within the VTA and the other within the ipsilateral nucleus accumbens (NACC). Dopamine (DA) levels in dialysates from both areas, as the index of the activity of mesolimbic DA neurons, were measured simultaneously. The results showed that intraperitoneal injection of ethanol at the doses of 1 and 2 g/kg increased extracellular DA concentrations in both the VTA and the NACC, suggesting increased DA neuronal activity. These ethanol-induced increases of the DA release in the VTA and the NACC were significantly attenuated by intra-tegmental infusion of SB 216641 (a 5-HT_{1B} receptor antagonist), but not BRL 15572 (a 5-$HT_{1D/1A}$ receptor antagonist) or WAY 100635 (a 5-HT_{1A} receptor antagonist). Administration of ethanol at the same doses did not significantly alter extracellular levels of GABA in the VTA. The results also showed that intra-tegmental infusion of CP 94253, a 5-HT_{1B} receptor agonist, significantly prolonged the effects of ethanol on NACC DA. The results suggest that blockade and activation of VTA 5-HT_{1B} receptors attenuates and potentiates the neurochemical effects of ethanol, respectively, and support the suggestion that VTA 5-HT_{1B} receptors may be involved in part in mediating the activating effects of ethanol on mesolimbic DA neurons.

Theme: Receptors
Topic: Serotonin receptor

Keywords: Ventral tegmental area; Nucleus accumbens; Ethanol; Dopamine; 5-HT_{1B} receptor; Dual-probe microdialysis

1. Introduction

It has been suggested that 5-hydroxytryptamine-1B (serotonin, 5-HT_{1B}) receptors play a role in modifying the reinforcing [15,43,53], intoxicating [15], and discriminative stimulus effects of ethanol [23,28] as well as regulating its voluntary intake [15,43,44]. Evidence supporting the role of 5-HT_{1B} receptors in mediating ethanol's effects comes from various studies. McBride et al. [44] investigated the densities of subtypes of 5-HT and dopamine (DA) receptors in the central nervous system (CNS) of alcohol-naïve alcohol-preferring P and alcohol non-preferring NP lines of rats. They found that there were no significant differences in the regional CNS densities of D_1, D_3, or 5-HT_3 binding sites between the P and NP lines. However, lower densities of 5-HT_{1B} receptors were found in the cortex, the septum, and the amygdala of the P lines compared with the NP lines. These results suggest that an innate alteration in the densities of 5-HT_{1B} receptors may be associated with the disparate alcohol-drinking behaviors of these two lines. Moreover, mice lacking the 5-HT_{1B} receptor gene consume more alcohol than wild-type mice and are more sensitive to some of ataxic

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effects of alcohol ([4,15], but see [17,27]). Using quantitative trait loci analysis in BXD recombinant inbred mice, the 5-HT_{1B} receptor gene has been identified as a potential candidate gene for alcohol preference [16]. Furthermore, polymorphisms in the 5-HT_{1B} receptor gene have been linked to antisocial personality and alcoholism in human ([39], but see [32]). The data cited above suggest that 5-HT_{1B} receptors might be one of the components in the genetic background underlying alcohol preference under some circumstances in rodents although recent research efforts have not consistently confirmed these original findings [17,27].

The involvement of 5-HT_{1B} receptors in alcohol-drinking behavior is further supported by the observations showing decreased alcohol consumption by 5-HT_{1B} agonists. Higgins et al. [30] reported that in rats that have been maintained on continuous access drinking, mCPP (a 5-HT_{1B} receptor agonist) suppressed alcohol intake during the first hour post-injection. In limited access drinking, mCPP produced dose-dependent suppression of alcohol intake in the rat. Wilson et al. [60] have tested the effects of TFMPP, a 5-$HT_{1B/2C/1A}$ receptor agonist [13], on ethanol ingestion and maintained behavior in an operant self-administration paradigm. They found that TFMPP produced a reduction in ethanol ingestion and maintained behavior at doses that failed to reduce locomotor activity. These results were consistent with the concept that activation of 5-HT_{1B} receptors may reduce ethanol intake and reinforced behavior. However, the contribution of 5-HT_{1A} and/or 5-HT_{2C} receptors to the TFMPP's actions cannot be ruled out in Wilson et al.'s study [60]. Recently, Tomkins and O'Neill [57] further explored the role of 5-HT_{1B} receptors in regulation of ethanol intake. They examined influences of the 5-$HT_{1B/1A}$ receptor agonist RU 24969 or CGS 12066B alone, RU 24969 plus GR 127935 (a 5-$HT_{1B/1D}$ receptor antagonists), and RU 24969 plus WAY 100135 or WAY 100635 (5-HT_{1A} receptor antagonists) on oral ethanol self-administration, respectively. The results showed that both RU 24969 and CGS 12066B significantly suppressed oral ethanol self-administration. Administration of GR 127935 significantly reversed the effects elicited by RU 24969, whereas neither WAY 100635 nor WAY 100135 had any effects. These data demonstrated that 5-HT_{1B} receptor activation suppressed oral ethanol self-administration and further supported the hypothesis that 5-HT_{1B} receptors play a role in regulating ethanol intake. Recently, 5-HT_{1B} receptors have also been found to be involved in mediation of the conditioned, or secondary reinforcing properties of ethanol when ethanol-paired conditioned stimulus paradigms were used [61].

The neurochemical mechanisms involved in the modulation of the alcohol-drinking behaviors by 5-HT_{1B} receptors are not known. Several lines of evidence suggest that DA neurons in the ventral tegmental area (VTA) are important for ethanol's reinforcing effects and self-administration. Thus, administration of ethanol increased the firing of DA neurons in the VTA [7,8,25] and enhanced somatodendritic DA release in the VTA [10] and terminal DA release in the nucleus accumbens (NACC) [26,59,63]. Moreover, alcohol-preferring P rats and Wistar rats self-administered ethanol directly into the VTA [24,54]. Previous studies also showed that VTA 5-HT_{1B} receptors may be involved in the modulation of activity of the mesolimbic DA neurons. Activation of VTA 5-HT_{1B} receptors has been reported to increase DA release in both the VTA and the ipsilateral NACC [64,67]. Therefore, a dopaminergic mechanism may be involved in 5-HT_{1B} receptor-mediated regulation of the alcohol-drinking behavior. That is, the reported suppression of alcohol intakes by 5-HT_{1B} receptor agonists may be due to the 5-HT_{1B} receptor activation-induced potentiation of ethanol's actions on mesolimbic DA neurons, therefore leading to lesser amounts of alcohol being consumed to produce the same CNS effects. The present study was designed to assess the involvement of VTA 5-HT_{1B} receptors in ethanol-induced increases in mesolimbic DA transmission. Towards this aim, dual-probe microdialysis was used with one in the VTA and the other in the ipsilateral nucleus accumbens (NACC) of freely moving Sprague–Dawley rats. Several serotonergic agents were administered into the VTA via retrograde microdialysis to minimize the effects of the compounds on the structures other than the VTA. The effects of the serotonergic manipulations on ethanol-induced increases of extracellular DA levels in the VTA and the NACC were assessed simultaneously. Since previous studies suggest that 5-HT_{1B} receptor-mediated activation of mesolimbic DA neurons may involve VTA GABAergic neurotransmission [67], extracellular GABA concentrations in the VTA were also monitored in some experiments to see whether ethanol affects VTA GABA levels as well.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 250–300 g at the time of surgery, were obtained from Harlan Sprague–Dawley Inc. (Indianapolis, IN, USA). They were housed at 21 ± 3 °C, 40–60% relative humidity, and maintained under 12:12-h light–dark conditions with ad libitum access to food and water. All animal care and experimentation were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

2.2. Drugs

WAY-100635 maleate {*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-2-pyridinyl-cyclohexanecarboxamide maleate}, BRL 15572 {4-[3-chlorophenyl]-α-[diphenyl-

methyl]-1-piperazineethanol hydrochloride} were purchased from Sigma (St. Louis, MO, USA). SB 216641 hydrochloride {*N*-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide hydrochloride} was obtained from Tocris (Ellisville, MO, USA). CP 94253 {5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-pyrrolo [3,2-b]pyridine} was generously provided by Pfizer (Groton, CT, USA). All drugs were dissolved in water and then diluted to desired concentrations with artificial cerebrospinal fluid (ACSF) before administration. Ethanol (Sigma) was administered intraperitoneally in a 20% solution (w/v) with 0.9% sterile saline. Other reagents used were of analytical grade.

2.3. Microdialysis

The animals were prepared for the microdialysis experiments as described in a previous paper [67]. In brief, surgery was conducted on a Kopf stereotaxic instrument under anesthesia with a combination of sodium pentobarbital (35 mg/kg ip) and halothane (5% in oxygen). Dialysis guide cannulae (Harvard Apparatus, Inc., S. Natick, MA, USA) were stereotaxically implanted over both the VTA and the ipsilateral NACC and attached to the skull with dental acrylic and machine screws. The coordinates relative to bregma and skull surface were as follows: the VTA: AP −5.2 mm, L 3 mm (at an angle of 14° from the sagittal plane to avoid rupture of the sagittal sinus), DV 8.0 mm, and the NACC: AP 1.7 mm, L 1.4 mm, DV 8.0 mm according to the Atlas of Paxinos and Watson [50]. The period of post-surgical recovery was at least 5 days. On the evening before the experimental day, each rat was placed in a Plexiglas chamber and dialysis probes (1 and 2 mm in length for the VTA and NACC, respectively), made from cellulose acetate hollow fibers (ID 215 ± 15 μm, molecular weight cutoff = 6000; Spectrum Medical Industries, Inc., Los Angeles, CA, USA), were inserted while gently restraining the freely behaving rat. Then, ACSF, which contained (in mM) Na^+ (150), K^+ (3.0), Ca^{2+} (1.2), Mg^{2+} (0.8), Cl^- (155), was perfused at 0.2 μl/min overnight. On the experimental day, the ACSF flow rate was increased to 2 μl/min. After 3–4 h, dialysate samples from both the VTA and NACC were collected at 20-min intervals into vials containing 5 μl 0.1 N HCl and stored at −80 °C until analysis. If VTA GABA was also measured, dialysate samples from the VTA were divided into two portions with one for measurements of DA and the other for determinations of GABA. Frozen samples showed no signs of degradation for up to 1 month in our previous studies.

The treatments for each experiment were as follows. Experiment 1: the rats were injected ip with saline (the same volume as ethanol, $n = 6$) or ethanol at the dose of 1 ($n = 7$) or 2 ($n = 7$) g/kg. Experiment 2: the rats received intra-VTA infusion of 10 μM SB 216641 (a 5-HT_{1B} receptor antagonist [29,52]), 10 μM BRL 15572 (a 5-$HT_{1D/1A}$ receptor antagonist [29,52]), or 10 μM WAY 100635 (a 5-HT_{1A} receptor antagonist [22]). Twenty minutes later, ethanol was administered intraperitoneally at the dose of 1 or 2 g/kg. The numbers of the animal in each treatment group were as follows: $n = 8$ (ethanol 1 g/kg + SB 216641), $n = 7$ (ethanol 1 g/kg + WAY 100635), $n = 7$ (ethanol 1 g/kg + BRL 15572), $n = 8$ (ethanol 2 g/kg + SB 216641), $n = 7$ (ethanol 2 g/kg + WAY 100635), and $n = 6$ (ethanol 2 g/kg + BRL 15572). Experiment 3: the rats received intra-VTA infusion of 10 μM CP 94253 (a 5-HT_{1B} receptor agonist [36]). Twenty minutes later, saline ($n = 6$) or ethanol at the dose of 1 ($n = 6$) or 2 ($n = 7$) g/kg was administered intraperitoneally.

In order to evaluate the implantation of the probe functionally, each dual-probe experiment was finished with infusion of 50 μM of baclofen, a $GABA_B$ receptor agonist, into the VTA probe and the response of extracellular DA in the ipsilateral NACC was determined. A significant decrease (≥50% deduction) in extracellular DA in the ipsilateral NACC after perfusion with baclofen was considered an appropriate implantation of the probe.

2.4. Analytical and histological procedure

For measurement of DA, dialysate samples were injected onto a high performance liquid chromatography (HPLC) system with electrochemical detection. This system consisted of an ESA solvent delivery system (model 580), an ESA microbore column (MD-150 × 1/RP-C18, 3 μM) or an ESA narrowbore column (MD-150 × 2/RP-C18, 3 μM), and an ESA coulochem II electrochemical detector equipped with a dual electrode analytical cell (Model 5041) and a guard cell (Model 5020). The guard cell was set at 400 mV, electrode at 175–200 mV with respect to palladium reference electrodes. A VICI micro-electric two-position valve actuator with a 5-μl (for the microbore column) or 50-μl (for the narrowbore column) injection loop was used for sample injection. The mobile phase contained 75 mM Na_2HPO_4, 1.53 mM sodium dodecyl sulfate, 25 μM EDTA, 100 μl/l triethylamine, 11.5% acetonitrile, and 11.5% methanol (pH 5.6 with H_3PO_4) and was pumped through the system at 0.07 (for the microbore column) or 0.25 (for the narrowbore column) ml/min. Chromatograms were integrated, compared with standards run separately on each experimental day, and analyzed using a computer-based data acquisition system (EZChrom Chromatography Data System, Scientific Software, Inc., San Ramon, CA, USA). The detection limit for dopamine was ~4 fmol at a 2:1 signal-to-noise ratio.

For determination of GABA, an isocratic HPLC system with electrochemical detection was used. This system consisted of an ESA solvent delivery system (model 580), an ESA autosampler (Model 542), a Waters Xterra™ MS column (50 × 3 mm, C18, 2.5 μM), and an ESA coulochem III electrochemical detector equipped with an analytical cell (Model 5011) and a guard cell (Model 5020). The guard cell was set at 650 mV, and the analytical cell at 250 mV (E1)

and 550 mV (E2). The mobile phase contained 100 mM Na_2HPO_4, 0.13 mM Na_2 EDTA, and 28% methanol (pH 6.4 with H_3PO_4) and was pumped through the system at 0.5 ml/min. Pre-column derivatization with *o*-phthaldialdehyde (OPA)/2-mercaptoethanol was performed automatically by the autosampler by mixing 15 μl of the working derivatizing reagent with 20 μl of dialysate samples or working standard solutions for 2 min. The detection limit for GABA was ~500 fmol at a 2:1 signal-to-noise ratio.

After completion of the dialysis, the animals were anesthetized with sodium pentobarbital and then intracardially perfused with buffered saline and 10% formalin prior to decapitation. The brains were removed quickly, and 40-μm thick coronal sections were cut on a freezing microtome, stained with neutral red, and analyzed in the light microscope. The heavy staining of gliosis along the guide cannula track permitted reliable location of the deepest point of penetration. A 2-mm-long (in the NACC) or 1-mm-long (in the VTA) dialysis membrane extended below the tip of the guide cannula. The point of the probe tip was then marked on coronal sections from the atlas of Paxinos and Watson [50].

2.5. Data analysis

Changes in dialysate DA and GABA induced by treatments were expressed as percentages of the baseline in each individual rat. The average DA or GABA levels in three samples immediately preceding the treatment was defined as the baseline (100%). The dialysis data were calculated as mean ± SEM and not corrected for the in vitro probe recovery. A two-way analysis of variance (ANOVA) followed by Tukey's tests was applied. All analyses were performed through computer-based software (SigmaStat). The criterion of significance was set at $P < 0.05$.

3. Results

Only data from animals with correct probe placements (at least 80% of the dialysis membrane in the desired areas) and appropriate accumbal DA responses to perfusion of the VTA with baclofen were included in data analyses. Approximately 70% of the animals that had undergone surgery had both probes correctly implanted in the VTA and NACC and met the functional criterion. Fig. 1 shows the placements of the probe tip in both the VTA and the NACC. The actual placements for the VAT and NACC probes are shown in Fig. 2.

3.1. Effects of systemic ethanol on extracellular DA and GABA in the VTA, and DA in the ipsilateral NACC

These experiments were necessary for this laboratory to establish our own effective doses of ethanol. After DA and GABA in the VTA, and DA in the ipsilateral NACC were stable, ethanol (1 and 2 g/kg) or saline was injected intraperitoneally and microdialysis was continued for another 2 h. The basal values (fmol/sample) of extracellular DA in the VTA and NACC were as follows: 9.04 ± 1.32 and 29.09 ± 3.09 (the saline group, $n = 6$), 10.99 ± 1.04 and 34.39 ± 2.72 (the 1 g/kg ethanol group, $n = 7$), and 8.94 ± 0.72 and 28.24 ± 2.30 (the 2 g/kg ethanol group, $n = 7$), respectively. There were no statistically significant differences in basal DA in the VTA or the NACC among the ethanol and saline groups. As shown in Fig. 3, intraperitoneal injection of saline had no significant effects on dialysate levels of DA in either the VTA or the ipsilateral NACC. However, administration of ethanol at the doses of 1 and 2 g/kg significantly increased extracellular DA concentrations by ~38% and ~56% of baseline in the VTA ($P < 0.01$ as compared with the saline group, the upper panel of Fig. 3) and ~51% and ~67% of baseline in the NACC ($P < 0.01$ as compared with the saline group, the lower panel of Fig. 3), respectively. As can be seen from this figure, the maximum increases in extracellular DA concentrations in the VTA were temporally correlated with those in the NACC after administration of ethanol at both doses.

Basal GABA levels (fmol/sample) in the VTA dialysate were as follows: 1729.95 ± 141.63 (the saline group, $n = 6$), 1791.73 ± 107.41 (the 1 g/kg ethanol group, $n = 7$), and 1647.43 ± 97.81 (the 2 g/kg ethanol group, $n = 7$). There were no statistically significant differences in the basal GABA levels among the ethanol and saline groups.

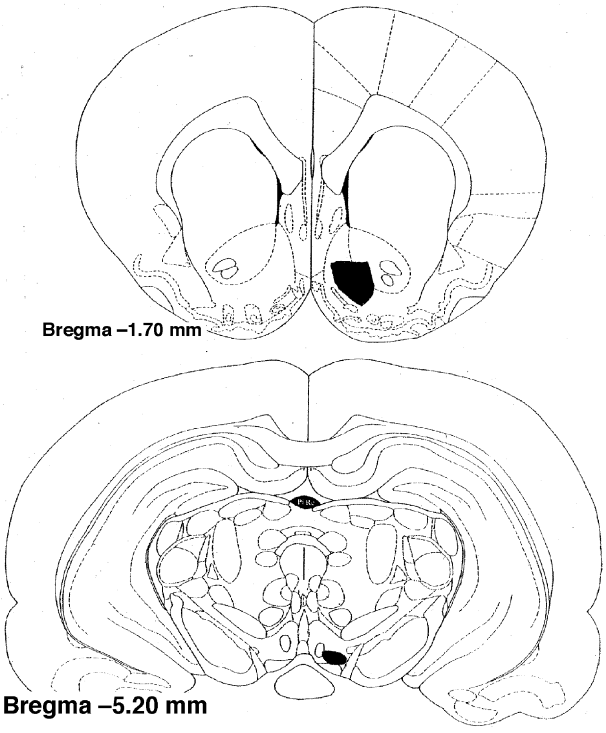

Fig. 1. Diagrammatic representation of microdialysis probe placements in the NACC (the upper panel) and the VTA (the lower panel). The shadowed area represents placements of the probe tips, but not the whole dialysis membrane.

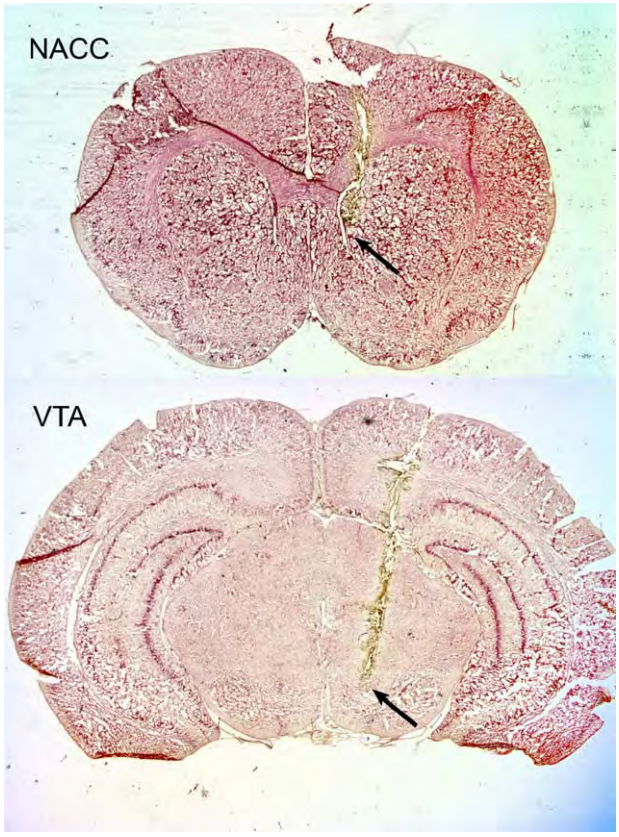

Fig. 2. Representative histological sections stained with neutral red which show tissue damage produced by a guide cannula positioned immediately dorsal to the NACC (the upper panel) or the VTA (the lower panel). A 2-mm-long (in the NACC) or 1-mm-long (in the VTA) dialysis membrane extends below the tip (as indicated by the arrow) of the guide cannula.

Administration of ethanol at the doses of 1 or 2 g/kg did not cause any significant changes in dialysate GABA levels as compared with the saline group (data not shown), suggesting that acute ethanol may not affect extracellular GABA in the VTA under the present experimental conditions. Since no measurable changes in VTA dialysate GABA were detected after administration of ethanol at the doses of 1 or 2 g/kg, GABA contents in the VTA dialysates were no longer assayed in the following experiments.

3.2. Effects of infusion of 5-HT-1 receptor antagonists into the VTA on ethanol-induced DA release in this region and in the ipsilateral NACC (Figs. 4 and 5)

Dual-probe microdialysis was the same as above except that a 5-HT-1 receptor antagonist was infused into the VTA 20 min before ethanol administration and remained throughout the experiments. The following antagonists were used: SB 216641, a 5-HT_{1B} receptor antagonist [29,52], BRL 15572, a 5-$HT_{1D/1A}$ receptor antagonist [29,52], and WAY 100635, and a 5-HT_{1A} receptor antagonist [22]. All these antagonists were infused into the VTA at the concentration of 10 μM in ACSF. The concentration of the antagonists was chosen based on the reports in the literature and ~ 5% of the efficiency of the probe used. It has been shown that 10–100 nM of WAY 100635 potently antagonized the 5-HT_{1A} receptor-mediated effects in the isolated guinea pig ileum [22]. Our previous results showed that local infusion of 10 μM SB 216641 significantly attenuated not only the effects of intra-tegmental CP 93129 on VTA DA and NACC DA but also on VTA GABA [67], suggesting that 10 μM of SB 216641 was sufficient to block VTA 5-HT_{1B} receptors. It has also been reported that the BRL 15572 at the concentration of 0.5 μM blocked the 5-HT_{1D} receptor-mediated effects on in vitro 5-HT release from the rat dorsal raphe nucleus slices [31]. Moreover, infusion of SB 216641, WAY 100635, or BRL 15572 at the concentration of 10 μM into the VTA for 2 h did not significantly alter extracellular DA levels in either the VTA or the ipsilateral NACC [67].

Figs. 4 and 5 show comparisons of ethanol (1 and 2 g/kg)-induced DA release in the VTA and the NACC in the presence and absence of SB 216641, WAY 100635, or BRL 15572. As shown in these figures, ethanol-induced DA releases, except those occurred in the VTA after 1 g/kg

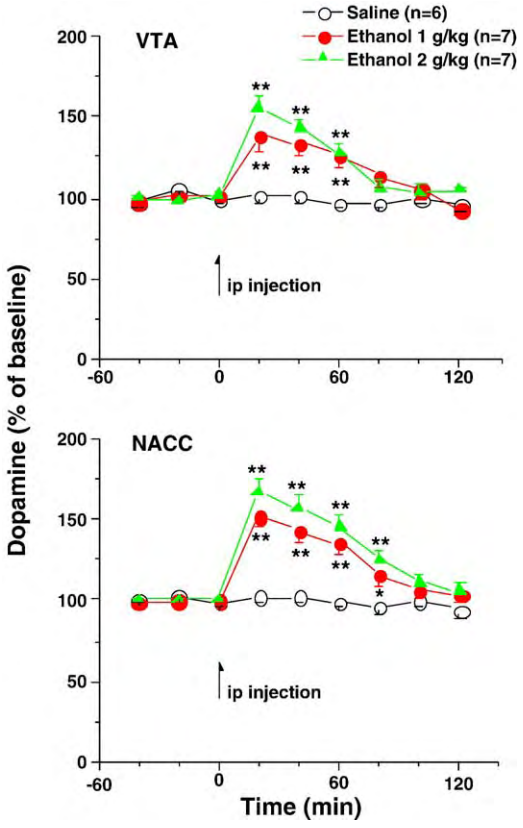

Fig. 3. Effects of acute ethanol on extracellular DA in the VTA (the upper panel) and the ipsilateral NACC (the lower panel). Microdialysis probes placed in the VTA and the NACC were perfused with ACSF simultaneously. Saline or ethanol (1 and 2 g/kg) was administered by intraperitoneal injection indicated by the arrow. Results are mean ± SEM from six to seven animals. *$P < 0.05$, **$P < 0.01$ as compared with the saline group (two-way ANOVA followed by Tukey's tests).

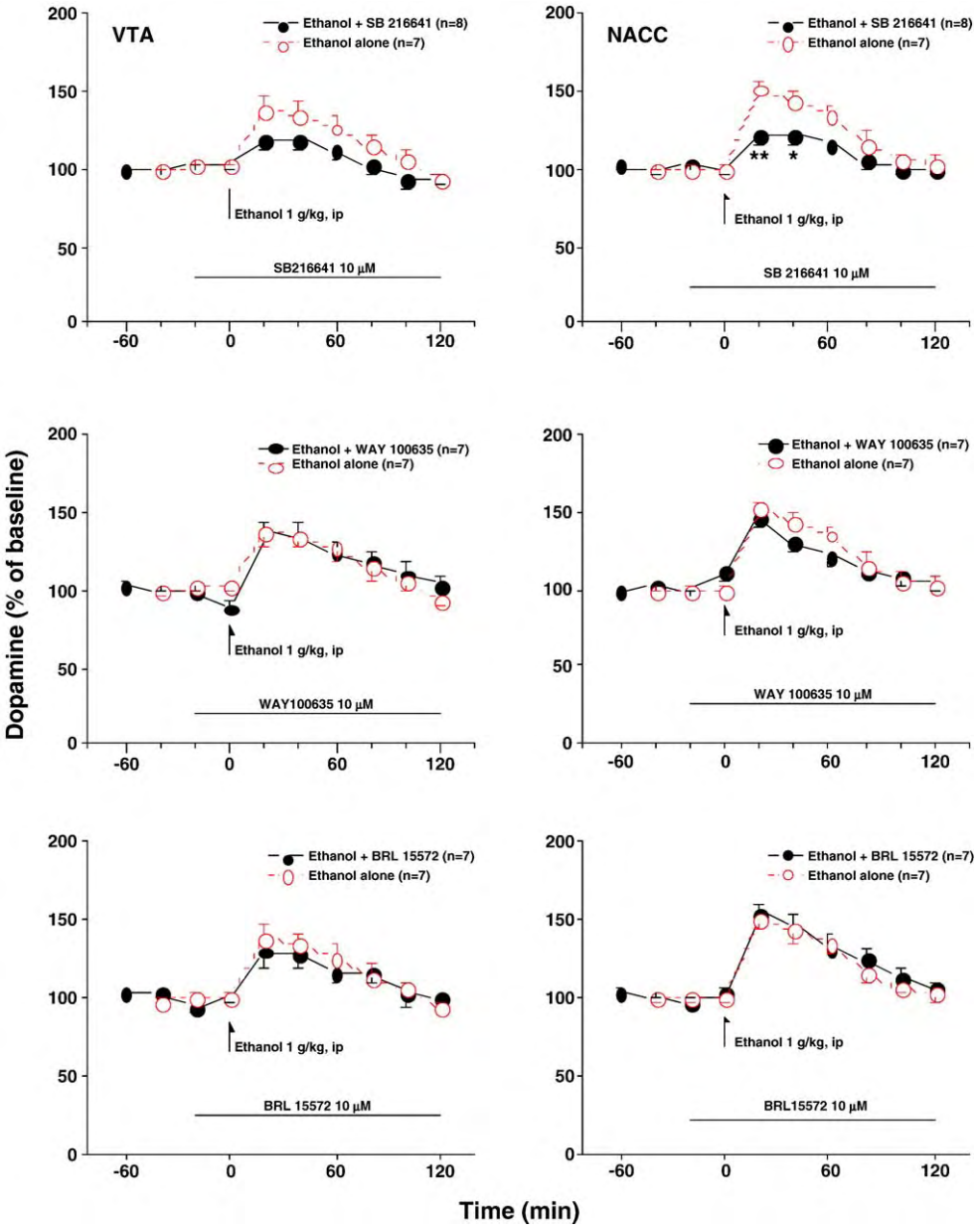

Fig. 4. Comparisons of ethanol (1 g/kg)-induced DA release in the VTA (the left panel) and the ipsilateral NACC (the right panel) in the presence and absence of SB 216641 (upper), WAY 100635 (middle), or BRL 15572 (lower). SB 216641 (10 μm), WAY 100635 (10 μm), or BRL 15572 (10 μm) was infused into the VTA 20 min before ethanol (1 g/kg ip) administration and remained throughout the experiments. Results are mean ± SEM from seven to eight animals. The data of the ethanol alone group were obtained from Fig. 3. The basal DA levels (fmol/sample) in the VTA and the NACC were as follows: 10.99 ± 1.04 and 34.39 ± 2.72 (the ethanol alone group, $n = 7$), 11.16 ± 0.81 and 46.78 ± 4.84 (the ethanol + SB 216641 group, $n = 8$), 10.31 ± 1.18 and 36.92 ± 3.96 (the ethanol + WAY 100635 group, $n = 7$), and 12.32 ± 1.37 and 37.71 ± 3.47 (the ethanol + BRL 15572 group, $n = 7$), respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests).

of ethanol (the upper left of Fig. 4), were all significantly attenuated by co-administration of SB 216641. In the presence of SB 216641, ethanol (1 and 2 g/kg)-induced NACC DA release (the upper right of Figs. 4 and 5) and ethanol (2 g/kg)-induced VTA DA release (the upper left of Fig. 5) were all significantly lower than those in the absence of SB 216641. However, co-administration of WAY 100635 or BRL 15572 had no significant effects on ethanol-induced DA release in either the VTA or the NACC.

3.3. Effects of infusion of CP 94253 into the VTA on ethanol (1 and 2 g/kg)-induced DA release in the ipsilateral NACC (Figs. 6 and 7)

These experiments were designed to investigate further the involvement of VTA 5-HT_{1B} receptors in modulation of the effects of ethanol on DA release in the NACC. Dual-probe microdialysis was the same as described in Section 3.1 except that CP 94253 (10 μM, a 5-HT_{1B} receptor agonist [36]) was infused into the VTA 20 min

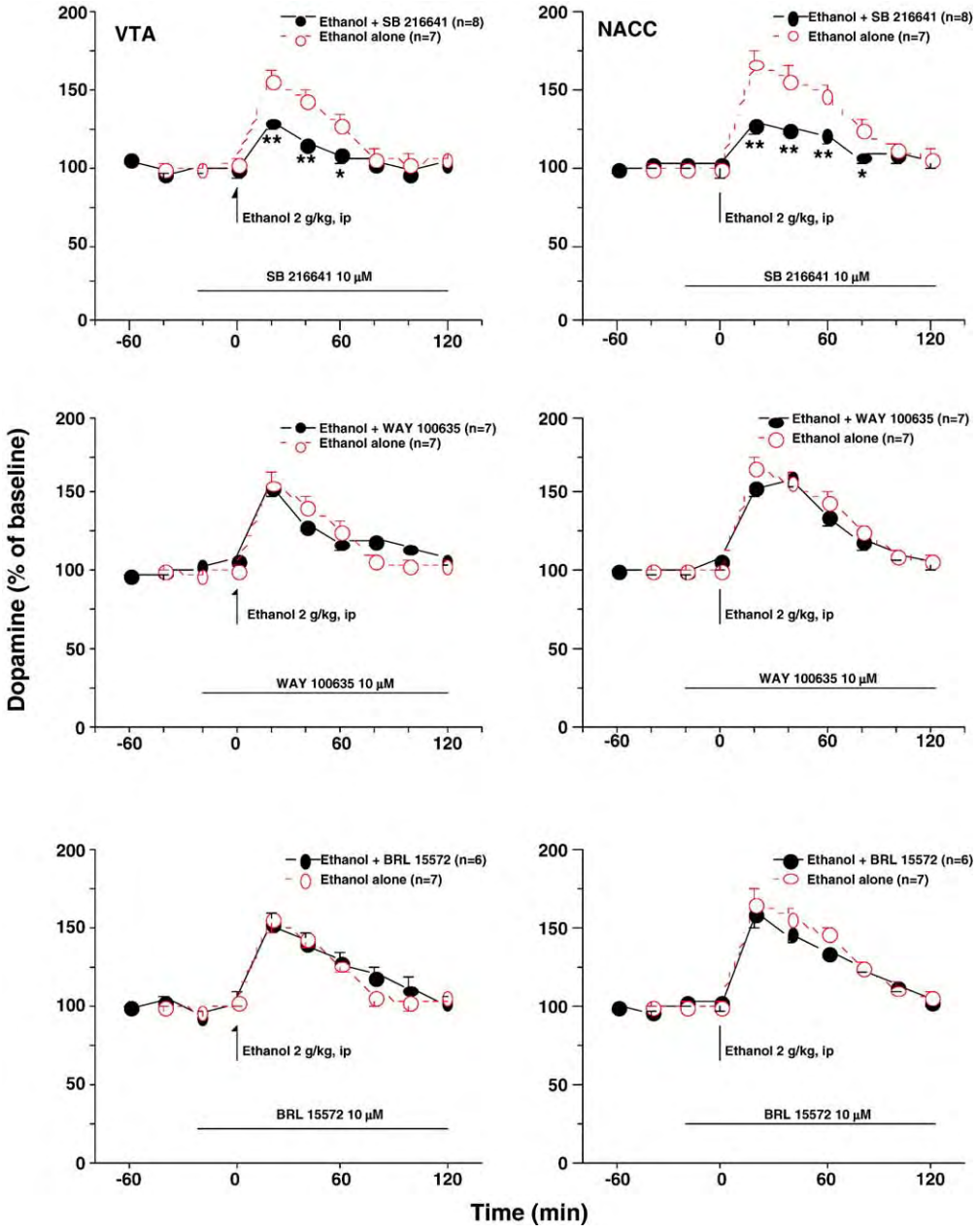

Fig. 5. Comparisons of ethanol (2 g/kg)-induced DA release in the VTA (the left panel) and the ipsilateral NACC (the right panel) in the presence and absence of SB 216641 (upper), WAY 100635 (middle), or BRL 15572 (lower). SB 216641 (10 μm), WAY 100635 (10 μm), or BRL 15572 (10 μm) was infused into the VTA 20 min before ethanol (2 g/kg ip) administration and remained throughout the experiments. Results are mean ± SEM from six to eight animals. The data of the ethanol alone group were obtained from Fig. 3. The basal DA levels (fmol/sample) in the VTA and the NACC were as follows: 8.94 ± 0.72 and 28.24 ± 2.30 (the ethanol alone group, $n = 7$), 8.24 ± 0.83 and 26.83 ± 2.53 (the ethanol ± SB 216641 group, $n = 8$), 9.25 ± 1.04 and 28.79 ± 2.20 (the ethanol + WAY 100635 group, $n = 7$), and 9.06 ± 0.93 and 27.64 ± 1.85 (the ethanol + BRL 15572 group, $n = 6$), respectively. *$P < 0.05$, **$P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests).

before ethanol injection and remained throughout the experiments. In a separate group of rats, perfusion of the VTA with CP 94253 (10 μM in ACSF) followed by the saline injection did not significantly alter extracellular DA levels in the ipsilateral NACC (Figs. 6 and 7). As shown in Figs. 6 and 7, however, administration of CP 94253 significantly changed the time course of extracellular DA concentrations following ethanol administration. In the absence of CP 94253, extracellular DA in the NACC increased rapidly to the maximum level after administration of ethanol at the doses of 1 (Fig. 6) and 2 g/kg (Fig. 7), then declined and reached the control level at 80 min after ethanol injection. However, in the presence of CP 94253, extracellular DA still remained significantly high levels as compared with either the saline or the ethanol alone group at 80–120 min after administration of ethanol at the both doses (Figs. 6 and 7). The results indicated that the treatment with CP 94253 significantly prolonged the

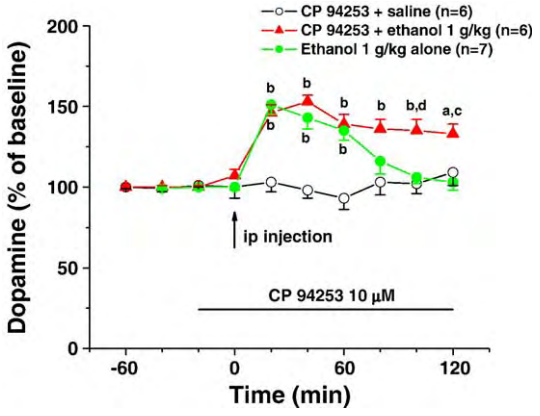

Fig. 6. Effects of co-administration of CP 94253 on ethanol (1 g/kg)-induced NACC DA release. CP 94253 (10 μM) was infused into the VTA through a probe as indicated by the bar. Ethanol (1 g/kg) or saline was injected intraperitoneally as indicated by the arrow. Extracellular DA in the ipsilateral NACC was measured by a second probe in this region. The data of the ethanol alone group was obtained from Fig. 3. $^{a}P < 0.05$, $^{b}P < 0.01$ as compared with the CP 94253 + saline group; $^{c}P < 0.05$, $^{d}P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests). The basal DA levels (fmol/sample) were as follows: 34.39 ± 2.72 (the ethanol alone group), 37.02 ± 3.64 (the CP 94253 + saline group), and 35.97 ± 3.73 (the CP 94253 + ethanol group).

effects of ethanol (1 and 2 g/kg) on the extracellular DA in the NACC although the maximum increases of NACC DA after co-administration of CP 94253 and ethanol did not significantly differ from those after administration of ethanol alone.

4. Discussion

Previous studies show that, in addition to classical DA release from synapses in their terminal areas, VTA DA neurons release DA from their somata and dendrites [1,11]. Accumulating evidence suggests that the release of DA in the VTA is regulated by mechanisms similar to those of axonal release although the former appears to be less sensitive to pharmacological manipulations. Thus, DA release in the VTA, similar to extracellular DA in the NACC, was augmented following potassium [35] or veratridine depolarization [33], and reduced after tetrodotoxin or calcium omission [10,12], suggesting that a depolarization-induced, exocytosis-mediated, somatodendritic release of DA occurred in the VTA under basal conditions. As a result, somatodendritic DA in the VTA, like that released from axon terminals in the NACC, can also be used as an index of the activity of mesolimbic DA neurons.

The present data show that systemic administration of ethanol at the doses of 1 and 2 g/kg increases extracellular DA concentrations not only in the NACC but also in the VTA in the same animal. Kohl et al. [37] also reported that systemic administration of ethanol at the doses of 2 and 3 g/kg simultaneously increased DA concentrations in the VTA and the ipsilateral NACC when assessed with dual-probe brain dialysis. Simultaneous elevations in the extracellular levels of DA in both cell body and terminal areas suggest that systemic administration of ethanol increases the firing rate of VTA DA neurons. Our results are in agreement with in vivo [25] and in vitro [7,8] electrophysiological experiments, and with previous microdialysis studies, which indicated that systemic administration of ethanol could increase the release of DA in the NACC [26,63] and the VTA [10] in separate animals. Interestingly, administration of nicotine, a drug that increases mesolimbic DA transmission by similar mechanisms as ethanol [45,47], also increased both somatodendritic DA release in the VTA and synaptic DA release in the NACC [68].

It has been reported that the excitatory action of ethanol on VTA DA neurons can be potentiated by 5-HT and 5-HT reuptake blockers [6,58]. This 5-HT-induced potentiation may be related to the stimulation of various 5-HT receptors. One of the 5-HT receptors suggested to have a modulatory effect on VTA DA neurons is the 5-HT_{1B} subtype. The main purpose of this study was to test the hypothesis that VTA 5-HT_{1B} receptors may modulate ethanol-induced increases in mesolimbic DA neurotransmission. This hypothesis was based on the observations that VTA 5-HT_{1B} receptors were involved in regulation of mesolimbic DA neurotransmission [64,67] and that local administration of ethanol into the VTA via a dialysis probe increased extracellular concentrations of 5-HT in this region [65], which could result in increased activation of VTA 5-HT_{1B} receptors.

To assess the involvement of 5-HT_{1B} receptors in ethanol's actions, WAY-100635, SB 216641, and BRL 15572 were used. The affinities (given as pK_i) of these

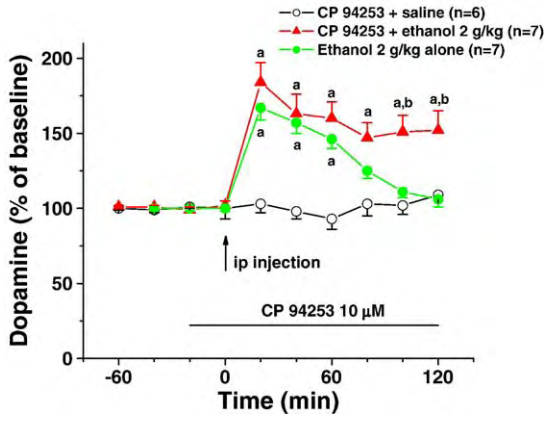

Fig. 7. Effects of co-administration of CP 94253 on ethanol (2 g/kg)-induced NACC DA release. CP 94253 (10 μM) was infused into the VTA through a probe as indicated by the bar. Ethanol (2 g/kg) or saline was injected intraperitoneally as indicated by the arrow. Extracellular DA in the ipsilateral NACC was measured by a second probe in this region. The data of the ethanol alone group were obtained from Fig. 3. The data of the CP 94253 + saline group were obtained from Fig. 6. $^{a}P < 0.01$ as compared with the CP 94253 + saline group; $^{b}P < 0.01$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey's tests). The basal DA levels (fmol/sample) were as follows: 28.24 ± 2.30 (the ethanol alone group), 37.02 ± 3.64 (the CP 94253 + saline group), and 34.99 ± 3.46 (the CP 94253 + ethanol group).

antagonists for 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptor subtypes are as follows: WAY 100635: 8.9, <6, and <6; SB 216641: 6.3, 9.0, and 7.6; BRL 15572: 7.7, 6.1, and 7.9 [29,52]. All these drugs were tested for their impacts on the actions of systemic ethanol on DA release in both the VTA and the NACC. The reason for which WAY 100635 was used is as follows. First, although SB 216641 is "selective" for the 5-HT_{1B} receptor, it may have weak antagonism at 5-HT_{1A} receptors when 10 μM is used. Moreover, the expression of 5-HT_{1A} receptors in the VTA has been demonstrated by autoradiographic [51] and in situ hybridization studies [62], providing anatomical evidence for potential interactions between 5-HT_{1A} receptors and VTA DA neurons. Indeed, it has been reported that administration of a 5-HT_{1A} receptor agonist has a biphasic effect on mesolimbic DA neurons with low doses being stimulatory and high doses being inhibitory on the neuronal firing rate [40,41]. As a result, the potential involvement of 5-HT_{1A} receptors in ethanol's actions needs to be addressed. If 5-HT_{1A} receptors are involved in the actions of ethanol, WAY 100635 (pK_i = 8.9 for the 5-HT_{1A} receptor), and BRL 15572 (pK_i = 7.7 for the 5-HT_{1A} receptor) would to some degree antagonize the effects of ethanol on VTA DA or NACC DA. However, this is not the case. The data presented here show that administration of neither WAY 100635 nor BRL 15572 into the VTA antagonizes the effects of systemic ethanol on extracellular DA in either the VTA or the NACC. These results are in opposition with the involvement of VTA 5-HT_{1A} receptors in ethanol's effects.

SB 216641 has been reported to have high affinity and selectivity for 5-HT_{1B} over 5-HT_{1D} receptors [46]. This drug shows more than 10-fold higher selectivity at 5-HT_{1B} (pK_i = 9.0) compared to 5-HT_{1D} (pK_i = 7.6) receptors [52]. BRL 15572 is recently identified as a selective 5-HT_{1D} receptor antagonist. It is more than 60-fold selective for 5-HT_{1D} over 5-HT_{1B} receptors [52]. It has been reported that CP 93129-induced inhibition of 5-HT release was antagonized by SB 216641 but remained unaffected in the presence of BRL 15572 [31]. The present data show that systemic ethanol-induced augmentations of VTA DA and NACC DA were significantly attenuated by local administration of SB 216641 but not by BRL 15572. These data are consistent with the involvement of VTA 5-HT_{1B} receptors, but not VTA 5-HT_{1D} receptors, in mediating the ethanol-induced excitation of VTA DA neurons.

In order to further investigate the involvement of VTA 5-HT_{1B} receptors in ethanol-induced activation of mesolimbic DA neurons, the 5-HT_{1B} receptor agonist CP 94253 was used. CP 94253 has at least a 40-fold and a 20-fold greater selectivities for the 5-HT_{1B} receptor over the 5-HT_{1A} and the 5-HT_{1D} receptor, respectively (K_i values are 89, 2, 860, 49, and 1600 nM for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, and 5-HT_2 receptors, respectively) [36]. The data presented here showed that local administration of 10 μM CP 94253 into the VTA did not significantly alter extracellular DA concentrations in the ipsilateral NACC. This result suggests that this concentration of CP 94253 may not cause sufficient activation of VTA 5-HT_{1B} receptors to produce significant increases in the activity of mesolimbic DA neurons under the present experimental conditions. However, this concentration of CP 94253 did enhance the effects of ethanol on extracellular DA in the NACC. The present data show that the treatment with CP 94253 significantly prolongs the effects of ethanol although it does not significantly enhance ethanol's peak effects. As shown in Figs. 6 and 7, extracellular DA remains significantly high at 80–120 min after ethanol in the presence of CP 94253 as compared with that in the absence of CP 94253. It was reported that the blood alcohol concentration (BAC) rose progressively to a peak at 20–40 min and then decreased thereafter, which was temporally correlated with changes of extracellular DA and 5-HT [63,69], following intraperitoneal injection of 1 or 2 g/kg of ethanol [19,42]. Therefore, our observation suggests that enhancement of ethanol's effects by CP 94253 occurs mainly during the descending limb of the BAC. Although the precise reasons for this type of enhancement are not presently understood, it is conceivable that this phenomenon may be due to additive or synergetic activation of VTA 5-HT_{1B} receptors by CP 94253 and released 5-HT by ethanol. The observed lack of further increases of ethanol's peak effects by CP 94253 may be a result of the VTA 5-HT_{1B} receptors already being maximally (or near maximally) activated by ethanol-evoked 5-HT, consequently, leading to little potential for further activation by CP 94253. Together with the data obtained with the 5-HT_{1B} receptor antagonist, the results support the suggestion that activation of 5-HT_{1B} receptors within the VTA may contribute to systemic ethanol-induced increases of mesolimbic DA neuronal activities.

The present results also show that although SB 216641 significantly attenuated ethanol-induced increases in DA release in both the VTA and NACC it did not completely block ethanol's effects. For example, injection of ethanol at the doses of 1 and 2 g/kg still caused NACC DA to increase to 121% (the upper right of Fig. 4) and 127% (the upper right of Fig. 5) of baseline, respectively, in the presence of SB 216641. This result may imply that, in addition to 5-HT_{1B} receptors, other receptors and/or mechanisms may also be involved in ethanol's neurochemical effects. Previous reports showed that local administration of a 5-HT_3 receptor antagonist antagonized systemic ethanol-induced increases of DA release in the VTA [10] or the NACC [9]. Moreover, the studies by Ericson et al. [18] and Blomqvist et al. [3] suggest that ethanol may elevate NACC DA via activation of VTA nicotinic acetylcholine receptors because ethanol-induced DA release in the NACC was antagonized by intra-tegmental administration of the acetylcholine receptor antagonist mecamylamine. Taken together, the results suggest that, in addition to 5-HT_{1B} receptors, multiple neurotransmitter receptors within the VTA may be involved in mediating the ethanol-evoked stimulation of mesolimbic DA neurons.

The mechanisms by which VTA 5-HT_{1B} receptors are involved in mediating ethanol's effects on mesolimbic DA transmission are unknown. It has been found that VTA DA neurons are under GABAergic inhibitory control [2]. Moreover, previous studies showed that activation of 5-HT_{1B} receptors inhibited high potassium-evoked [^{3}H] GABA release from rat VTA slices [66] and that GABA neurotransmission within the VTA may contribute to the 5-HT_{1B} receptor-mediated disinhibition of mesolimbic DA neurons [67]. These findings prompted us to speculate that the 5-HT_{1B} receptor-mediated inhibition of VTA GABA transmission may contribute to the stimulatory effect of ethanol on VTA DA neurons. Therefore, potential effects of systemic ethanol on VTA GABA were examined in this study. Unfortunately, the data presented here indicated that administration of ethanol at the dose of 1 or 2 g/kg did not significantly alter extracellular GABA in the VTA but did increased DA concentrations in both the VTA and the NACC. These results do not support our speculation. Cowen et al. [14] also reported that administration of ethanol (1 g) by gavage had no significant effects on extracellular GABA in the VTA or the substantia nigra measured by microdialysis. However, it should be pointed out that the technique of in vivo microdialysis requires long sampling time due to the low flow rates employed, and as such can only readily quantify substantial changes in release of neurotransmitters that maintained for a period of time. It is possible that administration of ethanol evokes a transient change in VTA GABA levels that is masked in a 20-min sample. In addition, previous microdialysis studies indicated that the part of GABA as measured by microdialysis did not fulfill the classical criteria for neuronal release [56], suggesting that GABA levels monitored by microdialysis probes may derive from non-neuronal pools in addition to the neuronal origin. Consequently, it is also possible that potential alterations in neuronal GABA release resulting from ethanol administration may only cause a small change in total extracellular GABA levels that cannot be detected by our HPLC system. As a result, an inability to detect measurable changes in the release of endogenous GABA in the VTA following ethanol does not necessarily mean that VTA GABA is not a target of acute ethanol. Further studies using more sensitive techniques to detect ethanol's effects on GABA in the VTA may be needed. In addition to regulating GABA release, 5-HT_{1B} heteroreceptors also provide an inhibitory modulation of glutamate release [46]. Glutamatergic afferents to the VTA are thought to play a role in regulating the activity of DA neurons in this region [5,34], and increased glutamatergic tone in the VTA has been reported to produce both excitatory and inhibitory effects on DA cell activity [21,55]. Thus, studies are currently underway in this laboratory to explore the possibility that 5-HT_{1B} receptor-mediated alterations in VTA glutamate efflux may be involved in part in the stimulatory effects of ethanol on VTA DA neurons.

The present study provides evidence that suggests that the VTA is one locus in the brain where 5-HT_{1B} receptors can modulate the neurochemical effects produced by peripherally administered ethanol. Since increased mesolimbic DA neurotransmission has been implicated in ethanol's rewarding effects [38], the present findings showing the involvement of VTA 5-HT_{1B} receptors in mediating ethanol's neurochemical effects may explain in part why 5-HT_{1B} receptors play a role in modifying the reinforcing [15,43,53], intoxicating [15], and discriminative stimulus effects of ethanol [23,28] as well as regulating its voluntary intake [15,43,44]. For example, the reported suppression of alcohol intakes by 5-HT_{1B} receptor agonists [30,57,60] may be caused, at least in part, by the drug-induced potentiation of ethanol's actions on mesolimbic DA neurotransmission. Interestingly, a recent study by O'Dell and Parsons [48] showed that VTA 5-HT_{1B} receptors also modulated cocaine-induced increases in NACC DA levels. This modulation of cocaine's neurochemical effects by 5-HT_{1B} receptors are consistent with the behavioral studies showing that 5-HT_{1B} receptors play a role in the regulation of cocaine's reinforcing and discriminative stimulus effects [20,49].

In summary, the present results indicate that activation and blockade of VTA 5-HT_{1B} receptors potentiates and attenuates the ethanol-induced increases in extracellular DA concentrations in both the VTA and the ipsilateral NACC, respectively. The data support the suggestion that VTA 5-HT_{1B} receptors may be involved in part in mediating the activating effects of ethanol on mesolimbic DA neurons.

Acknowledgments

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Further evidence that 5-hydroxytriptamine$_{1B}$ receptors modulate mesolimbic dopaminergic transmission and ethanol-induced increases in nucleus accumbens dopamine levels:

A study with 5-hydroxytriptamine$_{1B}$ knockout mice

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Abbreviations: ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; BAC, blood alcohol concentration; DA, dopamine; 5-HT, 5-hydroxytriptamine (serotonin); KO, knockout; NACC, nucleus accumbens; PCR, polymerase chain reaction; VTA, ventral tegmental area; WT, wild-type.

A recommended section: Neuropharmacology

Abstract

In order to investigate further the role of 5-hydroxytriptamine$_{1B}$ (5-HT$_{1B)}$ receptors in the modulation of mesolimbic dopamine (DA) transmission and ethanol's effects on this transmission, microdialysis with the probe in the nucleus accumbens (NACC) was performed in freely moving 5-HT$_{1B}$ knockout (KO) and their counterparts wild-type (WT) mice. The results showed that administration of RU 24969 (1 mg/kg, ip) increased NACC DA in the WT but not in the KO mice and that the RU 24969-induced increase in NACC DA in the WT mice was completely blocked by the pretreatment with SB 216641 (a 5-HT$_{1B}$ receptor antagonist, 0.1 mg/kg, ip) but not by WAY 100635 (a 5-HT$_{1A}$ receptor antagonist, 0.1 mg/kg, sc). The results also showed that systemic administration of 2 g/kg of ethanol produced more pronounced increases of NACC DA in the WT mice than in the KO mice. Moreover, the observed effects of ethanol on NACC DA were enhanced and attenuated by the pretreatments with RU 24969 (0.5 mg/kg, ip) and SB 216641 (0.1 mg/kg, ip), respectively, in the WT mice but remained unchanged in the KO mice. The present results obtained in transgenic mice are consistent with those previously obtained with the classic pharmacological technique, and further suggest that 5-HT$_{1B}$ receptors are involved in the modulation of not only mesolimbic DA transmission but also ethanol-induced increases in NACC DA levels.

Introduction

5-HT_{1B} receptors have been found to be involved in the regulation of mesolimbic dopaminergic transmission. Boulenguez et al. (1996) reported that administration of the 5-$HB_{1B/1A}$ receptor agonist RU 24969 at the doses of 0.1 - 2 mg/kg increased extracellular levels of DA in the rat NACC for more than 2.5 h. Consistent with this finding, Hallbus et al. (1997) demonstrated that the 5-HT-induced DA elevation in the NACC was markedly attenuated by co-perfusion of the 5-$HT_{1B/1D}$ receptor antagonist GR 127935. More convincing evidence suggesting the involvement of 5-HT_{1B} receptors in the regulation of mesolimbic DA transmission comes from recent studies with dual-probe microdialysis. These studies showed that direct administration of the 5-HT_{1B} receptor agonists CP 93129 {3-(1,2,5,6-tetrahydro-4-pyridyl)pyrrolo[3,2-b]pyrid-5-one} into the ventral tegmental area (VTA) increased not only somatodendritic DA release in this region (Yan et al., 2004) but also DA release from nerve terminals in the NACC (Yan and Yan 2001; O'Dell and Parsons, 2004; Yan et al., 2004), suggesting activation of the mesolimbic DA neurons. Moreover, the CP 93129-induced increases of extracellular DA in the VTA and the NACC were inhibited by the 5-HT_{1B} receptor antagonist SB 216641 but not by the 5-HT_{1A} receptor antagonist WAY 100635 or the 5-$HT_{1D/1A}$ receptor antagonist BRL 15572 (Yan and Yan, 2001; Yan et al., 2004). These data suggest that activation of 5-HT_{1B} receptors may be associated with enhanced mesolimbic DA transmission.

Evidence also suggests that 5-HT_{1B} receptors may be involved in mediating the stimulating effects of ethanol on mesolimbic DA neurons. Recent microdialysis studies carried out in this laboratory showed that increases of mesolimbic DA transmission by

systemic ethanol were significantly attenuated by co-administration of SB 216641 but not WAY 100635 or BRL 15572, and enhanced by co-administration of the 5-HT_{1B} receptor agonist CP 94253 {5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b]pyridine} (Yan et al., 2005). Since mesolimbic DA neurotransmission is important for ethanol's reinforcing effects and self-administration (Rodd et al., 2004), the observed involvement of 5-HT_{1B} receptors in ethanol's effects on mesolimbic DA neurons may in part explain why 5-HT_{1B} receptors play a role in modifying the reinforcing (Risinger et al., 1996) and discriminative stimulus effects (Grant and Colombo, 1993) of ethanol as well as regulating its voluntary intakes (Tomkins and O'Neill, 2000).

The data cited above were all obtained from the studies in which "selective" drugs that either block or activate 5-HT_{1B} receptors were employed. These agents, however, indeed were not truly specific for the 5-HT_{1B} receptor. For example, although CP 93129 is thought to be a specific 5-HT_{1B} receptor agonist, it also has to some extent affinities for 5-HT_{1A}, 5-HT_{1D}, and 5-HT_2 receptors (Macor et al., 1990). Thus, the possibility that the observed neurochemical changes after CP 93129 in the previous studies (Yan and Yan, 2001; Yan et al., 2004) might be the net result of changes resulting from the drug-induced activation of a number of different receptor subtypes rather than the result of activation of the 5-HT_{1B} receptor only cannot be completely ruled out. Clearly, further studies using different approaches are warranted to validate the results obtained with the classic pharmacological techniques.

Recently developed transgenic technology provides one method for investigating the contribution(s) of specific genes to complex behaviors. Using this technology, neuroscientists have successfully examined the contribution of several 5-HT receptor

subtypes to complex behaviors and neurotransmission through the generation of KO mice that lack the genes encoding these receptors. For example, 5-HT_{1B} receptor KO mice, achieved by homologous recombination, have been shown to be a useful tool for studying the role of 5-HT_{1B} receptors in various behaviors such as locomotion (Scearce-Levie et al., 1999), aggression (Saudou et al., 1994), sleep (Boutrel et al., 1999) and feeding (Lucas et al., 1998), in neurotransmission (Trillat et al., 1997), and in drug addiction such as cocaine (Rocha et al., 1998) and alcohol (Crabbe et al., 1996).

The aim of the present study was to investigate further the role of 5-HT_{1B} receptors in the regulation of mesolimbic DA transmission and in the ethanol's neurochemical effects. To this end, 5-HT_{1B} KO mice were used as a complementary approach to test further the following hypotheses that were derived from the above-cited studies using classic pharmacological techniques. One was that activation of 5-HT_{1B} receptors facilitated mesolimbic DA neurotransmission and the other was that 5-HT_{1B} receptors contributed to ethanol-induced increases of NACC DA. If the former hypothesis is correct, facilitation of mesolimbic DA transmission by activation of 5-HT_{1B} receptors would be absent in the KO mice. If the latter hypothesis is correct, it is likely that systemic ethanol would produce different effects on NACC DA in WT and KO mice and that administration of 5-HT_{1B} receptor agonists or antagonists would differentially affect ethanol-induced increases of NACC DA in WT and KO mice.

Method

Animals:

Breeding founders for 5-HT_{1B} KO and WT mice, both derived from the same 129/SV background (Knobelman et al., 2001), were kindly provided by Dr. Irwin Lucki from his established colonies at the University of Pennsylvania. Heterozygotes were produced by mating the KO founders with the WT founders. In order to ensure that subsequent mutants and controls have similar genetic background, KO and WT mice were generated by utilizing heterozygote breeding only (Phillips et al., 1999). All offspring was genotyped to verify that animals used in this study demonstrated the expected genetic deletion. This was confirmed in all cases. Mice were genotyped by the polymerase chain reaction (PCR) procedure. Briefly, tail was cut and DNA was extracted and purified using a Giagen DNeasy Tissue Kit (Valencia, CA) according to the protocol provided by the manufacturer. The Obtained tail DNA was used directly in the PCR reaction. The primers employed were the same as those used by Dr. Lucki (Knobelman et al., 2001) and as follows. NEO primer: CTT CTA TCG CCT TCT TGA CG; 5' 5-HT_{1B} receptor primer: GAC TTG GTT CAC GTA CAC AG; 3' 5-HT_{1B} receptor primer: CCC ATC AGC ACC ATG TAC AC. PCR amplification was performed on a T-gradient Thermocycler (Whatman Biometra) as follows: 94^0C 120 sec, then followed by amplification for 35 cycles (94^0C for 90 sec, 55^0C for 120 sec, and 72^0C for 120 sec), and a final step at 72^0C for 10 min. The PCR products were separated by electrophoresis in 1.5% agarose gel prepared in 1 x TBE (Tris-borate-EDTA) buffer containing 0.005% ethidium bromide. Expected sizes of PCR products were 560 bp for WT, 680 bp for KO, and 560 bp plus 680 bp for heterozygote mice. After weaning, KO or WT mice with the

same sex were housed three animals per cage, given free access to standard rodent chow and water, and maintained at 21 ± 3^0C, 40-60% relative humidity and under 12 h light/12 h dark conditions with lights on at 7:00 AM. The mice were ~ 12 weeks of age when used in this study and only males were employed. All animal care and experimentation were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

Drugs:

WAY-100635 maleate {N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-2-pyridinyl-cyclohexanecarboxamide maleate} was purchased from Sigma (St. Louis, MO., USA). SB 216641 hydrochloride {N-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1'-biphenyl]-4-carboxamide hydrochloride} and RU 24969 [5-methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1H-indole] were obtained from Tocris (Ellisville, MO., USA). All drugs were dissolved and diluted with sterile saline before administration. Ethanol (Sigma) was administered ip in a 20% solution (w/v) with 0.9% sterile saline. Other reagents used were of analytical grade.

Microdialysis:

Mice were anesthetized with a combination of sodium pentobarbital (35 - 40 mg/kg, ip) and halothane (5% in oxygen when needed) and positioned in a mouse stereotaxic instrument (Kopf Instruments, Tujunga, CA). Dialysis guide cannulae (CMA/7, CMA/Microdialysis, N. Chelmsford, MA) were stereotaxically implanted over one side of the NACC and attached to the skull with dental acrylic and machine screws. The coordinates relative to bregma and skull surface were as follows: AP 1.4 mm, L 0.8

mm and DV 5.0 mm according to the Atlas of Franklin and Paxinos (1997). The period of post-surgical recovery was at least 5 days. On the evening before the experimental day, each mouse was placed in a plexiglas chamber and dialysis probes (CMA/7, 1 mm in length, CMA/Microdialysis) were inserted while gently restraining the freely behaving mouse. On the experimental day, artificial cerebrospinal fluid (ACSF), which contained (in mM) Na^+ (150), K^+ (3.0), Ca^{2+} (1.2), Mg^{2+} (0.8), Cl^- (155), was perfused at 1.5 μl/min. After 3-4 h, dialysate samples were collected at 20-min intervals into vials containing 5 μl of 0.1 N HCl. After the basal DA release in the NACC was stable, treatments were administered and extracellular DA in the NACC was monitored after the treatments.

The treatments for each experiment were as follows. Experiment 1: KO or WT mice were injected ip with saline or RU 24969, a 5-$HT_{1B/1A}$ receptor agonist (Chopin et al., 1994), at the dose of 0.5 or 1 mg/kg. Experiment 2: KO or WT mice received injections of saline (ip), the 5-HT_{1B} receptor antagonist SB 216641 (Price et al., 1997) (0.1 mg/kg, ip), or the 5-HT_{1A} receptor antagonist WAY 100635 (Forster et al., 1995) (0.1 mg/kg, sc). Twenty minutes later, RU 24969 (1 mg/kg) was administered ip to each mouse. Experiment 3: KO or WT mice were injected ip with saline or ethanol at the dose of 1 or 2 g/kg. Experiment 4: RU 24969 (0.5 mg/kg) was injected ip to KO and WT mice, respectively. Twenty minutes later, ethanol at the dose of 1 or 2 g/kg was administered ip to each mouse. Experiment 5: SB 216641 (0.1 mg/kg, ip) was administered to KO and WT mice, respectively. Twenty minutes later, each mouse received injections of ethanol at the dose of 2 g/kg.

Analytical and histological procedure:

For measurement of DA**,** dialysate samples were injected onto a high performance liquid chromatography (HPLC) system with electrochemical detection. This system consisted of an ESA solvent delivery system (model 580), an ESA narrowbore column (MD-150 x 2/RP-C18, 3 μM), and an ESA coulochem II electrochemical detector equipped with a dual electrode analytical cell (Model 5041) and a guard cell (Model 5020). The guard cell was set at 400 mV, electrode at 175 - 200 mV with respect to palladium reference electrodes. A VICI micro-electric two-position valve actuator with a 50-μl injection loop was used for sample injection. The mobile phase contained 75 mM Na_2HPO_4, 1.53 mM sodium dodecyl sulfate, 25 μM EDTA, 100 μl/l triethylamine, 11.5% acetonitrile and 11.5% methanol (pH 5.6 with H_3PO_4), and was pumped through the system at 0.25 ml/min. Chromatograms were integrated, compared with standards run separately on each experimental day, and analyzed using a computer-based data acquisition system (EZChrom Chromatography Data System, Scientific Software, Inc., San Ramon, CA, USA). The detection limit for DA was ~ 4 fmol at a 2:1 signal-to-noise ratio.

After completion of the dialysis, the animals were anesthetized with sodium pentobarbital and then intracardially perfused with buffered saline and 10% formalin prior to decapitation. The brains were removed quickly, and forty-μm thick coronal sections were cut on a freezing microtome, stained with neutral red and analyzed under the light microscope. The heavy staining of gliosis along the guide cannula track permitted reliable location of the deepest point of penetration. A 1 mm-long dialysis membrane extended below the tip of the guide cannula. The point of the probe tip was

then marked on coronal sections from the atlas of Franklin and Paxinos (1997). Animals with incorrect probe placements were not included in the data analysis.

Data analysis:

Changes in dialysate DA induced by treatments were expressed as percentages of the baseline in each individual animal. The average DA levels in three samples immediately preceding the treatment was defined as the baseline (100%). The dialysis data were presented as mean ± S.E.M. and not corrected for the in vitro probe recovery. The overall effects of treatments on extracellular DA levels were analyzed by a two-way analysis of variance (ANOVA) with repeated measures over time. When the overall effects were found to reach significance ($P < 0.05$), further evaluations of the differences among the treatment groups at each time point or differences between the individual time points after the treatment and the corresponding baseline value were conducted by post hoc Tukey's or *t* tests. Evaluations of the difference in the basal DA values between the KO and WT mice and the difference in the basal DA values among the different treatment groups in either the KO or WT mice were conducted by *t* test and one-way ANOVA, respectively. All analyses were performed through computer-based software (SigmaStat). The criterion of significance was set at $P < 0.05$.

Result

1. Effects of systemic administration of RU 24969 on extracellular DA concentrations in the NACC of the KO and WT mice (Fig 1).

After basal DA in the NACC was stable, saline or RU 24969 (0.5 and 1 mg/kg) was injected ip to the KO or WT mice. The dose (0.5 and 1 mg/kg) of RU 24969 was chosen as administration of RU 24969 at this dose range increased NACC DA in rats (Boulenguez et al., 1996) and in C57BL/6J mice in our pilot study. The basal DA levels (fmol/sample, the volume of each sample was 30 μl, the same below) in the NACC were: 17.05 ± 2.99 (the KO saline group, n=6), 14.79 ± 1.9 (the KO RU 24969 0.5 mg group, n=6), 18.62 ± 2.03 (the KO RU 24969 1 mg group, n=6), 16.93 ± 2.53 (the WT saline group, n=6), 16.01 ± 1.62 (the WT RU 24969 0.5 mg group, n=6), and 17.45 ± 2.43 (the WT RU 24969 1 mg group, n=5). There were no statistically significant differences in the basal DA values between the KO and WT mice (P=0.972), nor were there differences among the saline, RU 24969 0.5 mg, and RU 24969 1 mg groups in the KO (P=0.937) or WT (P=0.423) mice.

As shown in Fig 1, administration of RU 24969 at the doses of 0.5 and 1 mg/kg did not produce significant changes in extracellular NACC DA in the KO mice as compared with the saline group (Panel A). In contrast, administration of RU 24969 at the same doses produced a dose-related increase in extracellular NACC DA in the WT mice (Panel B). There was a slight increase in NACC DA in the WT mice after 0.5 mg/kg of RU 24969 but the effect did not reach statistical significances as compared with the saline group (Panel B). However, administration of RU 24969 at the higher dose (1 mg/kg) produced statistically significant increases in NACC DA in the WT mice (Panel B). As

shown in Panel B, after administration of 1 mg/kg of RU 24969 extracellular NACC DA levels in the WT mice increased gradually, and statistically significant increases occurred from the time-points of 40 (as compared with the saline group) and 80 min (as compared with the 0.5 mg of RU 24969 group) until the end of the experiment.

2. Effects of pretreatment with SB 216641 or WAY 100635 on RU 24969-induced increases of NACC DA in the WT mice (Fig 2).

This experiment was performed to assess a possible role of 5-HT_{1A} receptors in the observed effect of 1 mg/kg of RU 24969 on NACC DA in the WT mice. To this end, WAY 100635 (0.1 mg/kg) and SB 216641 (0.1 mg/kg) were used. The dose (0.1 mg/kg) of WAY 100635 was chosen as this dose was able to completely block the effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT {(2R)-(+)-8-hydroxy-2-(di-n-propylamino)tetralin} but did not cause intrinsic changes in extracellular 5-HT when given alone (Knobelman et al., 2000). For SB 216641, the dose (0.1 mg/kg) was selected based on the following considerations. First, it has been reported that the effective receptor antagonist concentration of SB 216641 is similar to that of WAY 100635 (Rojas-Corrales et al., 2005). Second, the affinity of SB 216641 (pK_i = 9.0, Price et al., 1997) for the 5-HT_{1B} receptor is almost one order higher than that of RU 24969 (pK_i = 8.2, Chopin et al., 1994). Finally, previous studies showed that administration of the 5-$HT_{1B/1D}$ receptor antagonist GR 127935, a drug that is similar to SB 216641 in the affinity for the 5-HT_{1B} receptor (Pauwels, 1997), at the dose of as low as 0.056 mg/kg completely antagonized the effects of the 5-HT_{1B} receptor agonist CP 94253 on extracellular 5-HT concentrations, suggesting that 0.056 mg/kg of GR 127935 was sufficient to block 5-HT_{1B} receptors (Knobelman et al., 2000).

In this experiment SB 216641 (ip), WAY 100635 (sc) or saline (the same volume, ip) was administered 20 min before RU 24969 (1 mg/kg, ip). In a pilot study using separate groups of WT mice, it was found that administration of SB 216641 at 0.1 mg/kg or WAY 100635 at 0.1 mg/kg did not significantly alter NACC DA in the WT mice for as long as 2 h (data not shown).

The basal NACC DA levels were: 15.45 ± 2.06 (the saline plus RU 24969 group, n=5), 14.43 ± 1.58 (the SB 216641 plus RU 24969 group, n=6), and 13.69 ± 1.26 (the WAY 100635 plus RU 24969 group, n=6). There were no statistically significant differences in the basal DA values among these groups (P=0.418).

The effects of WAY 100635 or SB 216641 on RU 24969 (1 mg/kg, ip)-induced increases of NACC DA in the WT mice are shown in Fig 2. As can be seen from Panels A and B, the patterns of the time course of extracellular DA following RU 24969 were similar between the groups of saline plus RU 24969 (Panel A) and WAY 100635 plus RU 24969 (Panel B). Statistical analyses showed that there were no significant differences in extracellular DA concentrations after RU 24969 between these two groups (Panel D). These data suggest that pretreatment with WAY 100635 did not significantly alter the effects of RU 24969 on NACC DA in the WT mice. In contrast, however, pretreatment with SB 216641 completely blocked the ability of RU 24969 to increase NACC DA in the WT mice since no increases in extracellular DA concentrations were seen after RU 24969 in the SB 216641 plus RU 24969 group (Panel C). Statistical analyses showed that extracellular DA concentrations after RU 24969 were significantly lower in the SB 216641 plus RU 24969 group than those in either the saline plus RU 24969 group or the WAY 100635 plus RU 24969 group (Panel D). These data suggest

that pretreatment with SB 216641 antagonizes the effects of RU 24969 on NACC DA in the WT mice.

3. Effects of systemic ethanol on NACC DA in the KO and WT mice (Fig 3).

After basal DA in the NACC was stable, saline or ethanol at the dose of 1 or 2 g/kg was administered ip to KO or WT mice. The basal DA levels were: 13.72 ± 2.28 (the KO saline group, n=6), 15.08 ± 1.65 (the KO ethanol 1 g/kg group, n=7), 12.02 ± 1.12 (the KO ethanol 2 g/kg group, n=7), 13.6 ± 2.11 (the WT saline group, n=6), 14.09 ± 1.47 (the WT ethanol 1 g/kg group, n=8), and 13.25 ± 1.61 (the WT ethanol 2 g/kg group, n=7). There were no statistically significant differences in the basal DA values between the KO and WT mice (P=0.631), nor were there differences among the saline, ethanol 1 g/kg, and ethanol 2 g/kg groups in the KO (P=0.496) or WT (P=0.598) mice.

As shown in Fig 3, significant increases in NACC DA in the KO mice were observed only after administration of the higher dose (2 g/kg) of ethanol. The lower dose (1 g/kg) of ethanol caused a slight but not statistically significant increase in NACC DA in the KO mice (Panel A). However, administration of the same doses of ethanol all significantly increased NACC DA in the WT mice (Panel B). Comparisons of the NACC DA after 1 g/kg of ethanol show that there are no significant differences in any time points after ethanol between the KO and WT mice (Panel C). However, administration of ethanol at the dose of 2 g/kg produced more pronounced increases of NACC DA in the WT mice than in the KO mice. Statistical analyses show that significant differences in NACC DA were seen at the time points of 20, 60, 80, and 100 min after ethanol administration between the WT and KO mice (Panel D).

4. Effects of pretreatment with RU 24969 on ethanol-induced increases of NACC DA in the KO and WT mice (Fig 4).

This experiment compared the effects of the pretreatment with RU 24969 on ethanol-induced increases in NACC DA between the KO and WT mice. After basal DA in the NACC was stable, RU 24969 (0.5 mg/kg) was injected ip to the KO and WT mice, respectively. Twenty minutes later, each mouse received an ethanol injection (1 or 2 g/kg, ip) and extracellular DA in the NACC was monitored. The reason for choosing 0.5 mg/kg as the dose of RU 24969 in this experiment was as follows. The results from Experiment 1 showed that administration of RU 24969 at this dosage did not significantly alter NACC DA in either KO or WT mice, suggesting that 0.5 mg/kg of RU 24969 may not cause sufficient activation of the 5-HT_{1B} receptor to produce significant increases in mesolimbic DA transmission under the present experimental conditions. Using this sub-threshold dose of RU 24969, it may enable a potentiation of ethanol-induced increases of NACC DA to be detected easily.

The basal NACC DA levels in the KO mice were: 11.01 ± 1.44 (the group of RU 24969 + ethanol 1 g/kg, n=7) and 11.88 ± 1.40 (the group of RU 24969 + ethanol 2 g/kg, n=8); in the WT mice were: 10.96 ± 2.34 (the group of RU 24969 + ethanol 1 g/kg, n=7) and 12.46 ± 2.11 (the group of RU 24969 + ethanol 2 g/kg, n=7).

In pilot studies using separate groups of KO or WT mic, it was found that increases in NACC DA in the animal that received the first injection of saline followed by the second injection of 2 g/kg of ethanol 20 min later were similar in the magnitude to those in the animal that only received injections of the same dose of ethanol shown in Experiment 3, suggesting that pretreatment with saline did not significantly alter the

effects of subsequent ethanol on NACC DA in either the KO or WT mice. Thus, extracellular DA concentrations in the NACC after ethanol in the presence of the pretreatment with RU 24969 (this experiment) were compared with the corresponding values obtained in Experiment 3.

Fig 4 shows the effects of pretreatment with RU 24969 on the ethanol-induced increase of NACC DA in the KO and WT mice. As shown in Panels A and B, administration of RU 24969 did not significantly alter the effects of ethanol on NACC DA in the KO mice since extracellular DA concentrations were not significantly different between the ethanol alone group and the ethanol plus RU 24969 group at each time point after administration of ethanol at the dose of 1 (Panel A) or 2 g/kg (Panel B). In the WT mice, although pretreatment with RU 24969 did not significantly alter the effects of 1 g/kg of ethanol (Panel C), it did enhance the effects of 2 g/kg of ethanol on NACC DA (Panel D). As shown in Panel D, in the absence of RU 24969 extracellular DA in the NACC of the WT mice increased to the maximum level after administration of ethanol, then declined gradually and reached the baseline level at 120 min after ethanol injections. In contrast, however, in the presence of the pretreatment with RU 24969 extracellular DA still remained significantly high levels at 120-140 min after ethanol administration as compared with the ethanol alone group. The results suggested that the pretreatment with RU 24969 significantly prolonged the effects of 2 g/kg of ethanol on NACC DA in the WT mice.

5. Effects of pretreatment with SB 216641 on 2 g/kg of ethanol-induced increases of NACC DA in the KO and WT mice (Fig 5).

This experiment compared the effects of the pretreatment with SB 216641 on ethanol-induced increases in NACC DA between the KO and WT mice. Since administration of ethanol at the dose of 1 g/kg did not produce significant increases of NACC DA in the KO mice (Panel A of Fig 3), only 2 g/kg of ethanol was used.

SB 216641 (0.1 mg/kg) was injected ip to the KO and WT mice, respectively. Twenty minutes later, each mouse received an ethanol injection (2 g/kg, ip) and extracellular DA in the NACC was monitored. Extracellular DA concentrations in the NACC after ethanol in the presence of the pretreatment with SB 216641 (this experiment) were compared with the corresponding values obtained in Experiment 3. The basal NACC DA levels in these two groups were: 13.96 ± 2.11 (KO mice, n=6) and 12.82 ± 1.96 (WT mice, n=6). There were no significant differences in basal DA levels between the KO and WT mice (P=0.414).

As shown in Fig 5, pretreatment with SB 216641 did not significantly alter the effects of ethanol (2 g/kg) on NACC DA in the KO mice (the upper panel). However, ethanol (2 g/kg)-induced increases of NACC DA in the WT mice were significantly attenuated by the pretreatment with SB 216641. Comparisons of NACC DA in response to ethanol administration between the SB 216641 plus ethanol group and ethanol alone group show that extracellular DA was significantly lower in the former than in the latter at the time points of 20 and 60 min after ethanol injections.

Discussion

The present results show that there are no significant differences in the basal NACC DA levels between KO and WT mice. These data differ from earlier findings obtained with brain tissue preparations showing that tissue levels of DA in the NACC were decreased (-34%) in KO as compared with WT mice. It is well established that measures of DA tissue contents are not necessarily representative of DA levels in the extracellular space. For example, evidence for uncoupling between tissue and extracellular neurotransmitter concentrations comes from observations that 6-hydroxydopamine lesions of the NACC, which produce substantial deletions of DA as measured by whole-tissue assays, do not alter the extracellular levels of DA in the NACC unless the deletion is severe (>90%) (Robinson et al., 1990). Therefore, although the previous tissue and present extracellular data seem paradoxical, they are not irreconcilable. Our data also differ from previous studies by Shippenberg et al. (2000) who showed that basal extracellular DA levels in the NACC were significantly higher in KO mice than in WT mice. This discrepancy between this and Shippenberg et al.'s studies may be caused in part by the difference in methodology. For example, basal DA levels were measured by conventional microdialysis in this study but by the zero net flux method of quantitative microdialysis in Shippenberg et al.'s studies.

The data presented here also show that administration of RU 24969 at the dose of 1 mg/kg increases NACC DA in WT mice but not in KO mice. Augmentation of NACC DA in WT mice by RU 24969 is consistent with the data obtained in normal rats showing that administration of RU 24969 increased extracellular DA concentrations in the NACC (Boulenguez et al., 1996) and the striatum (Galloway et al., 1993). The finding that

facilitation of mesolimbic DA transmission by RU 24969 is present in WT mice but absent in 5-HT_{1B} KO mice suggests that activation of 5-HT_{1B} receptors may be associated with the observed effects of RU 24969 on NACC DA.

To assess further the involvement of 5-HT_{1B} receptors in RU 24969's effects, WAY 100635 and SB 216641 were used. Their affinities (given as p*K*i) for 5-HT_{1A} and 5-HT_{1B} receptor subtypes are: WAY 100635: 8.9 and < 6; SB 216641: 6.3 and 9.0 (Price et al., 1997; Forster et al., 1995). Both drugs were tested for their impacts on the action of systemic RU 24969 on DA release in the NACC. The reason for which WAY 100635 was used is as follows. First, RU 24969 is a 5-$HT_{1B/1A}$ receptor agonist. Therefore, a role of 5-HT_{1A} receptors in the observed effect of RU 24969 on NACC DA in the WT mice cannot be ruled out. Moreover, the expression of 5-HT_{1A} receptors in the mesolimbic DA system has been demonstrated by autoradiographic (Pazos and Palacios, 1985) and in situ hybridization studies (Wright et al., 1995), providing anatomical evidence for potential interactions between 5-HT_{1A} receptors and mesolimbic DA transmission. Indeed, it has been reported that administration of 5-HT_{1A} receptor agonists has biphasic effects on mesolimbic DA neurons with low doses being stimulatory and high doses being inhibitory on the neuronal firing rate (Lejeune et al., 1997; Lejeune and Millan, 1998). Furthermore, it has been reported that compensatory changes in sensitivity of 5-HT_{1A} receptors occur in certain brain areas of 5-HT_{1B} KO mice. Altered sensitivity of 5-HT_{1A} receptors may dramatically change the response to 5-HT_{1A}-related drugs such as RU 24969 in 5-HT_{1B} KO mice. Consequently, the observed absence of RU 249696-induced facilitation of NACC DA in the KO mice might not be due to the deletion of 5-HT_{1B} receptors but due to changed sensitivity of 5-HT_{1A} receptors. As a result, a potential

involvement of 5-HT_{1A} receptors in the observed effects of RU 24969 needs to be addressed. If 5-HT_{1A} receptors are involved in the actions of RU 24969, WAY 100635 would to some extent antagonize the effects of RU 24969 on NACC DA. However, this is not the case. The data presented here show that pretreatment with WAY 100635 did not antagonize the effects of RU 24969 as compared with the saline plus RU 24969 group. In sharp contrast to WAY 100635, however, the effects of RU 24969 on NACC DA in WT mice were completely blocked by the 5-HT_{1B} receptor antagonist SB 216641. These data suggest that it is 5-HT_{1B} but not 5-HT_{1A} receptors that are involved in RU 24969's effects in the WT mice. Together with the data showing that RU 24969 increases NACC DA only in the WT but not in the KO mice, the results suggest that activation of 5-HT_{1B} receptors is associated with increased mesolimbic DA transmission. The present data obtained from the transgenic animals extend and confirm the previous studies obtained with the classic pharmacological approach, and provide additional support to the hypothesis that activation of 5-HT_{1B} receptors facilitates mesolimbic DA neurotransmission.

The present study also shows that systemic administration of ethanol at the dose of 2 g/kg produced more pronounced augmentations of NACC DA in WT mice than in KO mice. One consideration in the interpretation of differential responses of NACC DA to systemic ethanol is the possibility of genetic differences in pharmacokinetic processes. However, this possibility seems unlikely because 5-HT_{1B} KO and WT mice do not differ in ethanol elimination after intraperitoneal exposure to ethanol. The previous studies by Crabbe et al. (1996) showed that acute clearance of ethanol did not differ (0.49 ± 0.06 mg ethanol/ml blood/h for the KO mice and 0.50 ± 0.08 for the WT mice) during 4 hours

after ip injections of 3.5 g/kg of ethanol. Interestingly, KO and WT mice do not differ in cocaine metabolism, either. It has been reported that the blood and brain levels of cocaine and its metabolites benzoylecgonine and norcocaine are identical in 5-HT_{1B} KO mice when compared with WT mice (Rocha et al., 1998). Therefore, it is very possible that the observed differences in ethanol-induced increases of NACC DA between the KO and WT mice may be due to the presence and absence of 5-HT_{1B} receptors in these genotypes.

This hypothesis is further supported by the findings showing that pretreatment with RU 24969 enhanced the effects of ethanol in WT mice but not in KO mice. Using a sub-threshold dose of RU 24969, we showed that pretreatment with RU 24969 did not alter the effects of ethanol on NACC DA in the KO mice but did enhance the effects of 2 g/kg of ethanol in the WT mice. The data presented here show that the pretreatment with RU 24969 significantly prolongs the effects of ethanol although it does not significantly enhance ethanol's peak effects. As shown in Panel D of Fig 4, extracellular DA remains significantly high at 120–140 min after ethanol in the presence of RU 24969 as compared with that in the absence of RU 24969. It was reported that the blood alcohol concentration (BAC) rose progressively to a peak at 20-40 minutes and then decreased thereafter following intraperitoneal injection of 1 - 2 g/kg of ethanol (Ferraro et al., 1990). Therefore, our observation suggests that enhancement of ethanol's effects by RU 24969 occurs mainly during the descending limb of the BAC. Although the precise reasons for this type of enhancement are not presently understood, it is conceivable that this phenomenon may be due to additive or synergetic activation of 5-HT_{1B} receptors by RU 24969 and released 5-HT by ethanol. The observed lack of further increases of ethanol's peak effects by RU 24969 may be a result of the 5-HT_{1B} receptors already

being maximally (or near maximally) activated by ethanol-evoked 5-HT, consequently, leading to little potential for further activation by RU 24969. The present data showing enhancement of NACC DA by activation of 5-HT_{1B} receptors in the WT mice but not in the 5-HT_{1B} KO mice provide additional support to the hypothesis that activation of 5-HT_{1B} receptors may contribute to ethanol-induced increases of mesolimbic DA transmission.

If the hypothesis that 5-HT_{1B} receptors are involved in ethanol's effects is correct, it is likely that administration of 5-HT_{1B} receptor antagonists would produce different effects on ethanol-induced increases of NACC DA in KO and WT mice. In order to further investigate the involvement of 5-HT_{1B} receptors in ethanol's effects, SB 216641 was used. The data presented here showed that pretreatment with SB 216641 significantly attenuated the effects of ethanol in the WT mice but not in the KO mice. Together with the data obtained with RU 24969, the results further support the hypothesis that 5-HT_{1B} receptors may be involved, at least in part, in the effects of ethanol on mesolimbic DA transmission.

The present results show that administration of 2 g/kg of ethanol increases NACC DA in the KO mice and this increase is still present following SB 216641 treatment. Our data also show that although SB 216641 significantly attenuated ethanol-induced increases in NACC DA in the WT mice it did not completely block ethanol's effects. For example, injection of ethanol at the dose of 2 g/kg still caused NACC DA to increase to 122% of baseline in the presence of SB 216641 in the WT mice (the lower panel of Fig 5). These results may imply that, in addition to 5-HT_{1B} receptors, other receptors and/or mechanisms may also be involved in ethanol's neurochemical effects. Previous reports

showed that local administration of 5-HT_3 receptor antagonists antagonized systemic ethanol-induced increases of DA release in the VTA (Campbell et al., 1996) and the NACC (Campbell and McBride, 1995). Moreover, the studies by Ericson et al. (2003) and Blomqvist et al. (1997) suggest that ethanol may elevate NACC DA via activation of VTA nicotinic acetylcholine receptors because ethanol-induced DA release in the NACC was antagonized by intra-tegmental administration of the acetylcholine receptor antagonist mecamylamine. Taken together, the results suggest that, in addition to 5-HT_{1B} receptors, multiple neurotransmitter receptors within the mesolimbic DA pathway may be involved in mediating the ethanol-evoked stimulation of mesolimbic DA neurons. Because the present studies involved systemic administration, they cannot determine the location of the 5-HT_{1B} receptor responsible for the modulation of the effects of ethanol on mesolimbic DA transmission.

Using transgenic animals, the present study provides further evidence suggesting that 5-HT_{1B} receptors can modulate the neurochemical effects produced by peripherally administered ethanol. The involvement of 5-HT_{1B} receptors in ethanol-induced increases of NACC DA may be one of mechanisms underlying the 5-HT_{1B} receptor-mediated regulation of alcohol intakes. That is, the reported suppression of alcohol intakes by 5-HT_{1B} receptor agonists (Tomkins and O'Neill, 2000) may be due to the receptor activation-induced potentiation of ethanol's actions on mesolimbic DA transmission, therefore, leading to lesser amounts of alcohol being consumed to produce the same central nervous system effects. Interestingly, recent studies by O'Dell and Parsons (2004) showed that 5-HT_{1B} receptors also modulated cocaine-induced increases in NACC DA levels. This modulation of cocaine's neurochemical effects by 5-HT_{1B} receptors are

consistent with the behavioral studies showing that 5-HT_{1B} receptors play a role in the regulation of cocaine's reinforcing and discriminative stimulus effects (Filip et al., 2003).

In summary, the present results obtained in transgenic mice are consistent with those previously obtained with the classic pharmacological technique, and further suggest that 5-HT_{1B} receptors are involved in the modulation of not only mesolimbic DA transmission but also ethanol-induced increases in NACC DA levels.

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Footnotes to the title

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Legend

Fig 1. Effects of RU 24969 on extracellular DA concentrations in the NACC of KO (Panel A) and WT (Panel B) mice. RU 24969 (0.5 or 1 mg/kg) or saline was injected ip as indicated by the arrow. Results, expressed as the percentage of baseline values, are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ as compared with the saline group; $^{++}$ $P < 0.01$ as compared with the RU 24969 0.5 mg group (two-way ANOVA followed by Tukey's tests). For basal DA values in each group, see the text.

Fig 2. Effects of pretreatment with WAY 100635 or SB 216641 on RU 24969-induced increases of NACC DA in the WT mice. Panels A, B, and C show the time course of NACC DA after pretreatments with saline (Panel A), WAY 100635 (0.1 mg/kg, Panel B), and SB 216641 (0.1 mg/kg, Panel C) followed by 1 mg/kg of RU 24969 twenty minutes later, respectively. * $P < 0.05$, ** $P < 0.01$ as compared with the baseline value (two-way ANOVA followed by Tukey's tests). Panel D shows comparisons of NACC DA after RU 24969 among the groups of saline plus RU 24969, WAY 100635 plus RU 24969, and SB 216641 plus Ru 24969. * $P < 0.05$, ** $P < 0.01$ as compared with the saline plus Ru 24969 group; $^{++}$ $P < 0.01$ as compared with the WAY 100635 plus RU 24969 group (two-way ANOVA followed by Tukey's tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. For basal DA values in each group, see the text.

Fig 3. Effects of systemic ethanol on NACC DA in the KO and WT mice. Ethanol (1 or 2 g/kg) or saline was injected ip as indicated by the arrow. Panels A and B show the time courses of extracellular DA in the NACC following saline or ethanol injections in the KO (Panel A) and WT (Panel B) mice, respectively. ***** $P < 0.05$, ****** $P < 0.01$ as compared with the saline group; $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$ as compared with the 1 g/kg ethanol group (two-way ANOVA followed by Tukey tests). Panels C and D show comparisons of NACC DA between the KO and WT mice in response to ethanol at the doses of 1 (Panel C) and 2 g/kg (Panel D), respectively. ***** $P < 0.05$ as compared with the KO mice (two-way ANOVA followed by Tukey tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. For basal DA values in each group, see the text.

Fig 4. Effects of pretreatment with RU 24969 on ethanol-induced increases of NACC DA in the KO (Panels A and B) and WT (Panels C and D) mice. RU 24969 (0.5 mg/kg) was injected ip as indicated by the arrow. Twenty minutes later, ethanol at the dose of 1 or 2 g/kg was administered indicated by the second arrow. The data of the ethanol alone group in each panel were obtained from Fig 3. ***** $P < 0.05$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. For basal DA values in each group, see the text.

Fig 5. Effects of pretreatment with SB 216641 on ethanol-induced increases of NACC DA in the KO (the upper panel) and WT (the lower panel) mice. SB 216641 (0.1 mg/kg) was injected ip to KO or WT mice. Twenty minutes later, each animal received ip injections of 2 g/kg of ethanol. Extracellular DA concentrations in the NACC after

ethanol were compared between the groups of ethanol alone and ethanol plus SB 216641 in the KO (the upper panel) and WT (the lower panel) mice. The data of the ethanol alone group were obtained from Fig 3. ***** $P < 0.05$ as compared with the ethanol alone group (two-way ANOVA followed by Tukey tests). Results, expressed as the percentage of baseline values, are mean ± S.E.M. For basal DA values in each group, see the text.

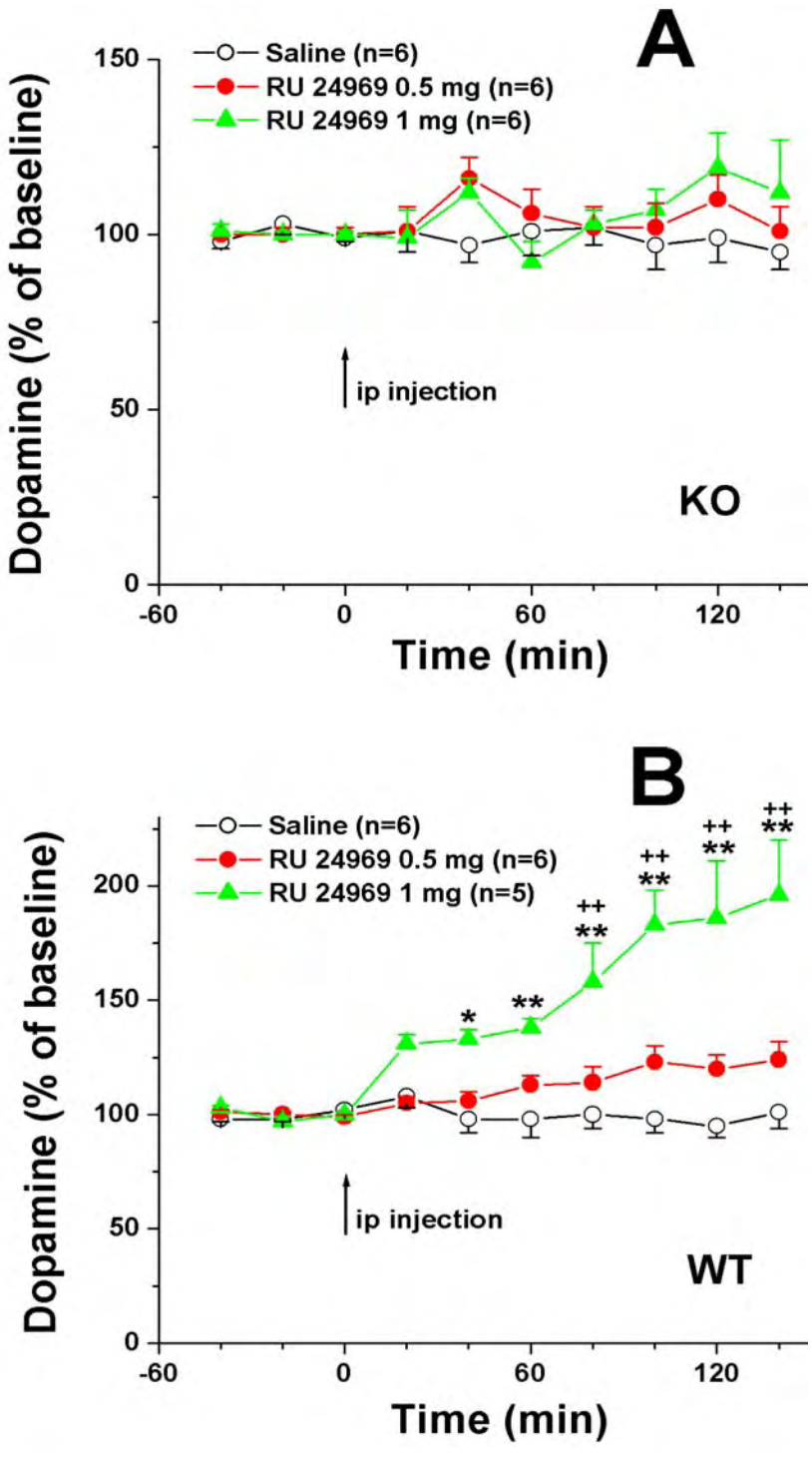
A
Saline (n=6)
RU 24969 0.5 mg (n=6)
RU 24969 1 mg (n=6)
Dopamine (% of baseline)
150
100
50
0
ip injection
KO
-60
0
60
120
Time (min)
B
Saline (n=6)
RU 24969 0.5 mg (n=6)
RU 24969 1 mg (n=5)
Dopamine (% of baseline)
200
150
100
50
0
*
**
++
**
++
**
++
**
ip injection
WT
-60
0
60
120
Time (min)

Fig 1

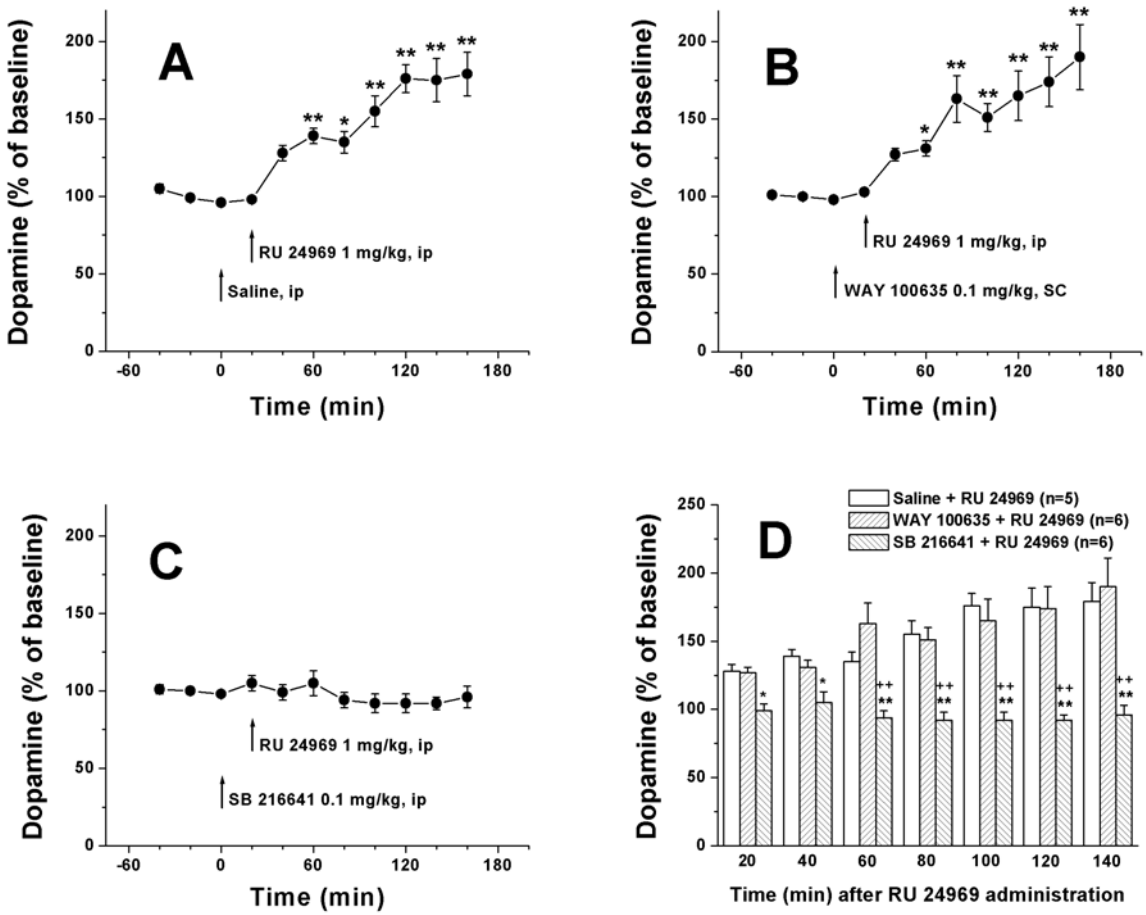
A
Dopamine (% of baseline)
Time (min)
RU 24969 1 mg/kg, ip
Saline, ip
B
Dopamine (% of baseline)
Time (min)
RU 24969 1 mg/kg, ip
WAY 100635 0.1 mg/kg, SC
C
Dopamine (% of baseline)
Time (min)
RU 24969 1 mg/kg, ip
SB 216641 0.1 mg/kg, ip
D
Saline + RU 24969 (n=5)
WAY 100635 + RU 24969 (n=6)
SB 216641 + RU 24969 (n=6)
Dopamine (% of baseline)
Time (min) after RU 24969 administration

Fig 2

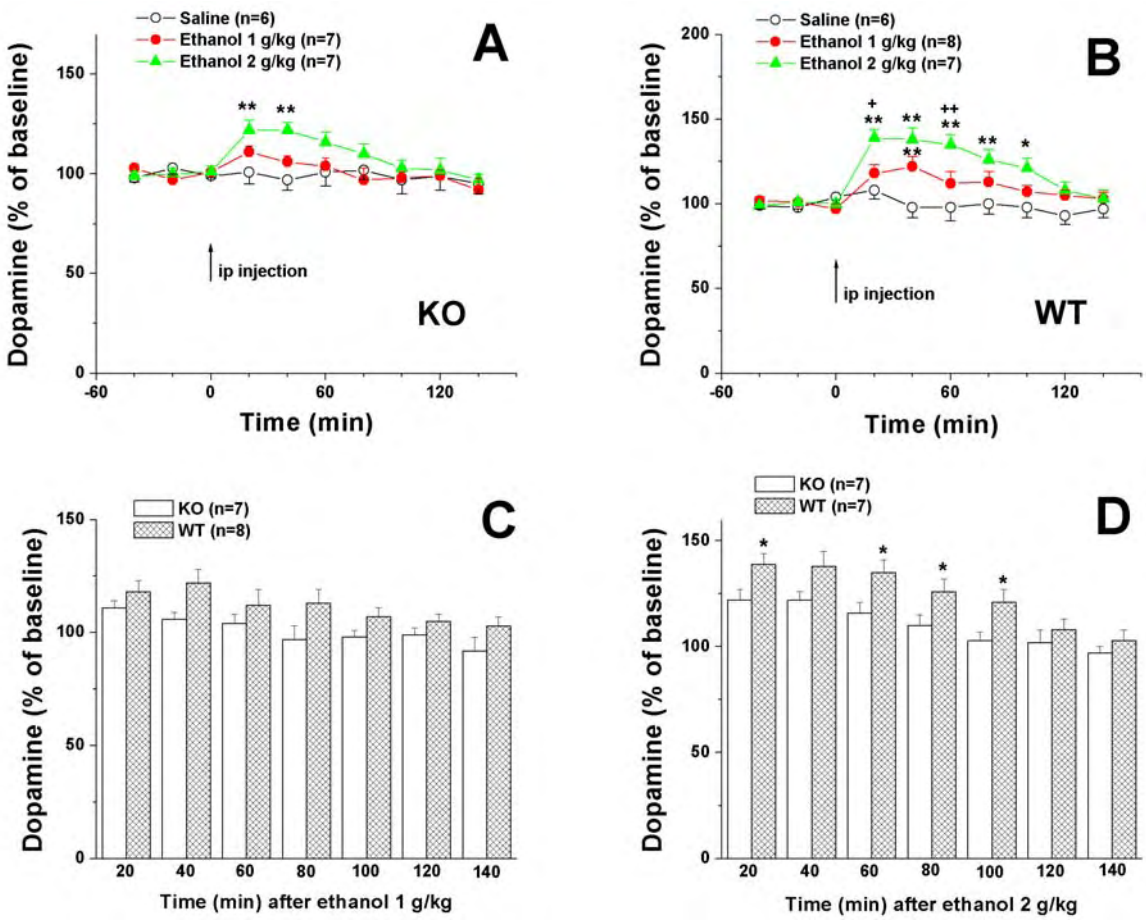
A
Saline (n=6)
Ethanol 1 g/kg (n=7)
Ethanol 2 g/kg (n=7)
Dopamine (% of baseline)
Time (min)
ip injection
KO
B
Saline (n=6)
Ethanol 1 g/kg (n=8)
Ethanol 2 g/kg (n=7)
Dopamine (% of baseline)
Time (min)
ip injection
WT
C
KO (n=7)
WT (n=8)
Dopamine (% of baseline)
Time (min) after ethanol 1 g/kg
D
KO (n=7)
WT (n=7)
Dopamine (% of baseline)
Time (min) after ethanol 2 g/kg

Fig 3

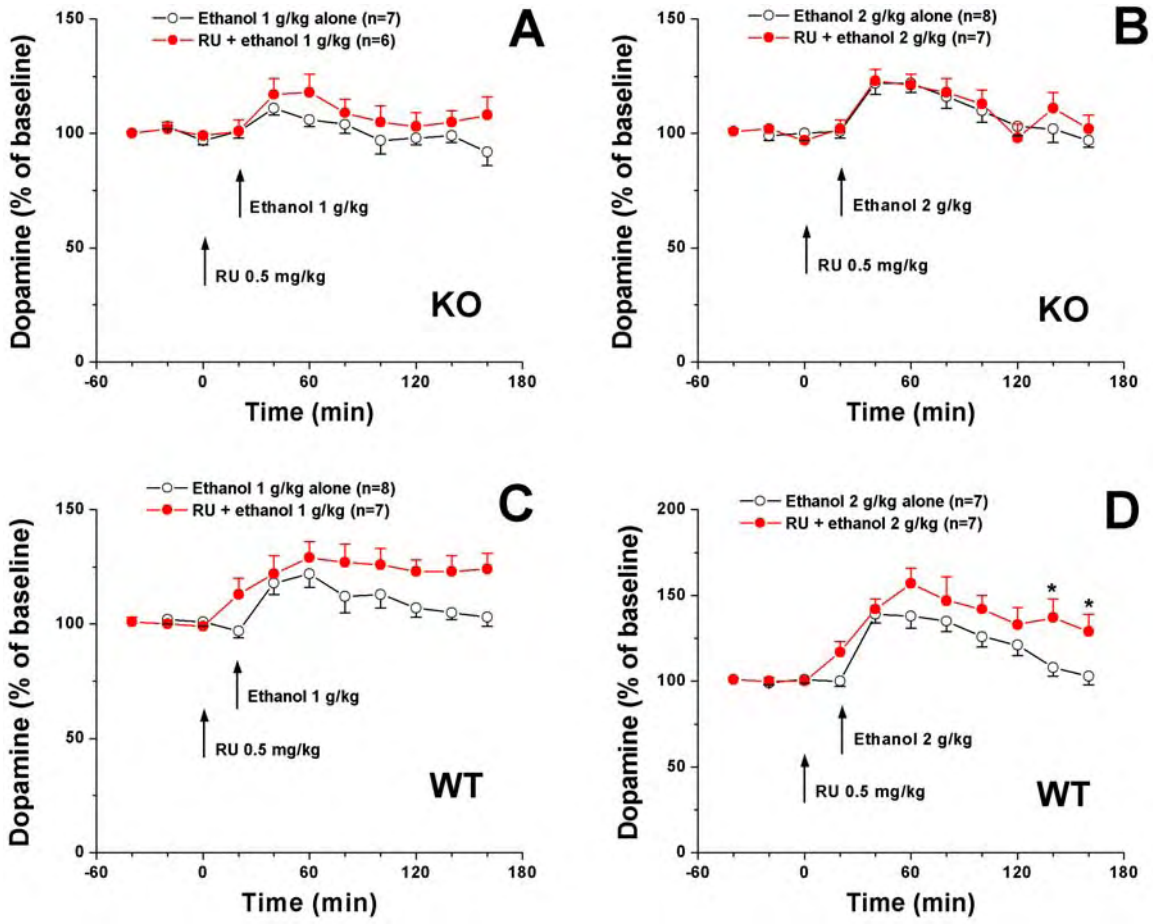
A
Ethanol 1 g/kg alone (n=7)
RU + ethanol 1 g/kg (n=6)
Ethanol 1 g/kg
RU 0.5 mg/kg
KO
Dopamine (% of baseline)
Time (min)
B
Ethanol 2 g/kg alone (n=8)
RU + ethanol 2 g/kg (n=7)
Ethanol 2 g/kg
RU 0.5 mg/kg
KO
C
Ethanol 1 g/kg alone (n=8)
RU + ethanol 1 g/kg (n=7)
Ethanol 1 g/kg
RU 0.5 mg/kg
WT
D
Ethanol 2 g/kg alone (n=7)
RU + ethanol 2 g/kg (n=7)
Ethanol 2 g/kg
RU 0.5 mg/kg
WT

Fig 4

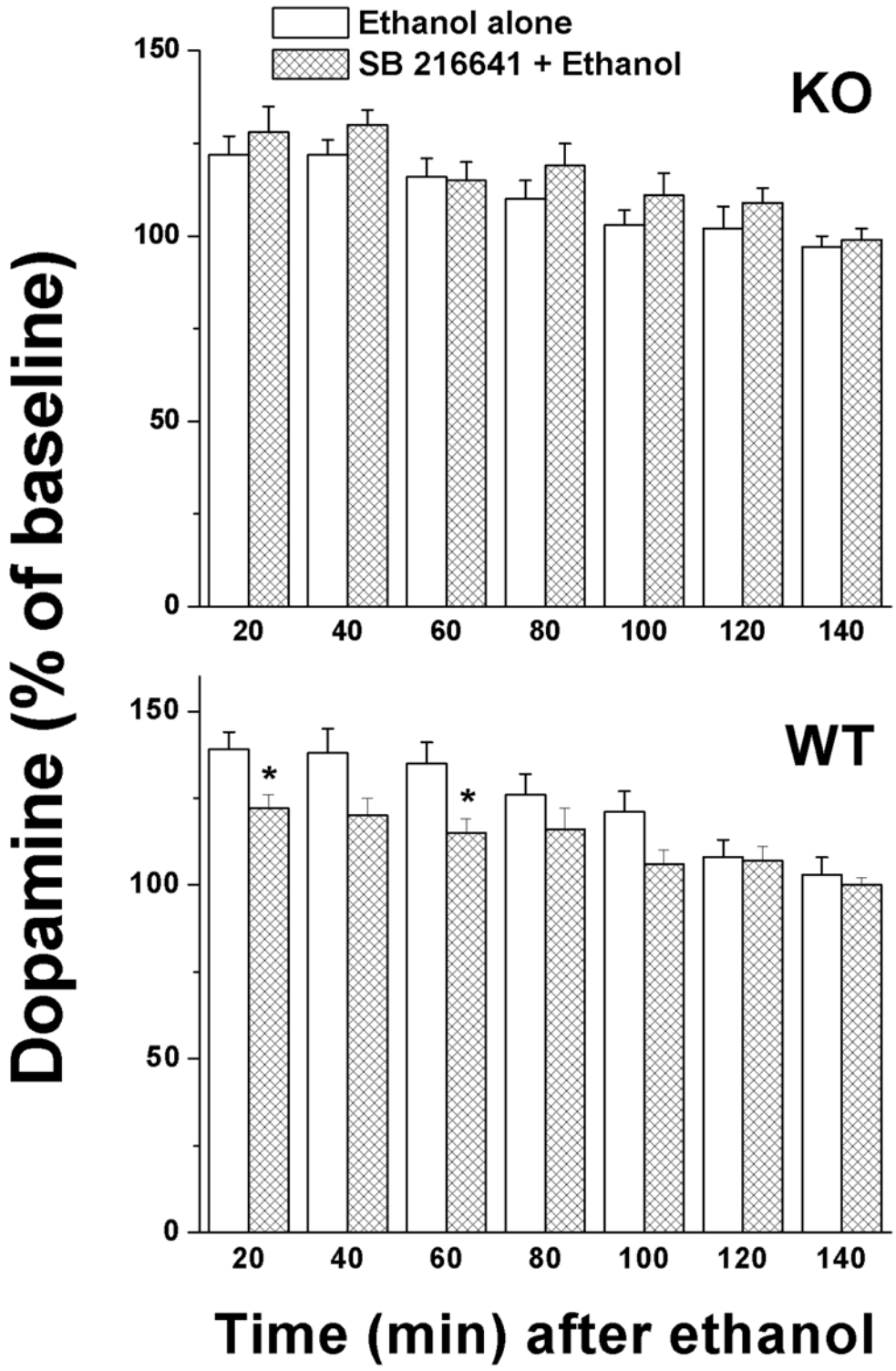
Ethanol alone
SB 216641 + Ethanol
KO
WT
Dopamine (% of baseline)
Time (min) after ethanol
150
100
50
0
20
40
60
80
100
120
140
*
*

Fig 5